ANTIMICROBIAL RESISTANCE OF *SALMONELLA*, *ESCHERICHIA COLI* AND *CAMPYLOBACTER* FROM PIGS ON-FARM IN ALBERTA AND SASKATCHEWAN CANADA

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By

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ABSTRACT

This cross-sectional study described antimicrobial resistance (AMR) in healthy pigs in 20 Alberta and Saskatchewan herds. All herds used antimicrobials; the daily probability of antimicrobial exposure was 0.8 for nursery pigs and 0.3 for grow-finish pigs. *Salmonella* spp. (n = 468) were isolated from nursery, grow-finish pigs and sows while *Escherichia coli* (n = 1439) and *Campylobacter* spp. (n = 405) were isolated from grow-finish pigs.

Fifty-nine percent of the *Salmonella* were pansusceptible. Isolates from sows were more likely to be pansusceptible than those from other production phases, while *Salmonella* from nursery pigs were more likely to be multiresistant. All *Salmonella* and *E. coli* were susceptible to ceftriaxone and ciprofloxacin, drugs critically important to human medicine, while one *E. coli* was resistant to ceftiofur. Resistance was most common to tetracycline and sulfamethoxazole (*Salmonella,* 35% and 27%; *E. coli*, 68% and 46%). Although often considered an 'indicator' organism, *E. coli* AMR was a poor sentinel for *Salmonella* AMR at the herd-level.

Antimicrobial resistance genes, described in 151 *E. coli,* were associated in two sets: *aadA1 / sul1* / *tetA* and bla_{TEM} / *strA – strB / sul2* / *tetB*. Associations between genes consistently matched associations between phenotypes suggesting phenotype data may be useful for predicting co-selection. Demonstrating dose-response relationships between

various antimicrobial exposures and resistance phenotypes in *E. coli* reiterated the importance of co-selection. Significant predictors included exposures in other production phases and to unrelated drugs. Four *E. coli* resistance-phenotypes were associated with macrolide exposure; the most commonly used antimicrobial class in study herds. Additionally, 70% of the *Campylobacter* were resistant to a macrolide and this resistance was associated with macrolide exposure in nursery pigs. Study herds did not use quinolones. Despite this, 15% of *Campylobacter* were resistant to a quinolone. Both *Campylobacter* and *E. coli* AMR clustered within herds, indicating on-farm interventions could mitigate AMR in pigs.

This study described AMR in enteric bacteria from healthy pigs. Identifying dose-response relationships between antimicrobial resistances and exposures to unrelated drugs, and exposures of pigs in different production phases, emphasize the importance of judicious antimicrobial use in pig production.

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Completing my degree has been both challenging and interesting; I could not have achieved this goal without the help and support of so many people. First, my sincere appreciation goes to my supervisor, Dr. Cheryl Waldner. Cheryl, thank you for all you have taught me, for your patience and your mentorship. You always went beyond the call of duty. To the members of my graduate committee, Dr. Richard Reid-Smith, Dr. John Harding, and Dr. Trisha Dowling, thank you for all of the time, effort and guidance you each gave me. Dr. Carruthers, thanks for keeping me ‘on-track’ and following ‘protocol’.

Finally, I would like to thank those people at WCVM who have helped to make this experience so fantastic. To my teachers, mentors and fellow graduate students in Large Animal Clinical Sciences thank you for all the support and everything you have taught me.

Dedication

I dedicate this thesis to my family: To my husband Colin, thank you for your love and encouragement to follow my dreams. It means everything that you always believe in me. To my son, Tim, you have changed my life in ways I could never have imagined. And to my parents, thank you for instilling your love of learning in me.

ORIGINAL CONTRIBUTION

This thesis presents the findings of an independent research project. I contributed extensively to all aspects of this research project, from conception to final presentation. I was involved in the project design and in preparing and submitting grant proposals. Obtained funding was used for fecal sample and antimicrobial use data collection and laboratory analysis. My responsibilities included enrolling over half of the study herds and collecting over half of the samples and antimicrobial use data. The remaining herds, samples and data were obtained from Dr. Wendy Wilkins. Her research project "Evaluation of the Performance of Culture, Danish-Mix ELISA and PCR for Detection of *Salmonella* in Swine" ran concurrent with this project which allowed us to collaborate. I coordinated all sample submissions to the laboratories, entered all antimicrobial use data and conducted all data analyses described in this thesis.

I did not perform any of the laboratory analysis. The Agri-Food Laboratories Branch (AFLB), Food Safety Division, Alberta Agriculture and Food, Edmonton, Alberta; Laboratory Services Division (LSD), University of Guelph, Guelph, Ontario; and Prairie Diagnostic Services (PDS), Saskatoon, Saskatchewan cultured fecal samples for *Salmonella*. *Salmonella* antimicrobial susceptibility testing was perfomed by AFLB and PDS. *Salmonella* serotyping was conducted by the Saskatchewan Health Provincial Laboratory, Regina, Saskatchewan and both serotyping and phagetyping were performed by the Office International des Épizooties Reference Laboratory for *Salmonellosis* in

Guelph, ON. Prairie Diagnostic Services conducted the *Escherichia coli* cultures and both AFLB and PDS tested the *E. coli* isolates for antimicrobial susceptibility. The *E. coli* antimicrobial resistance gene detection was performed by the Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec while the virulence gene testing was conducted by PDS. *Campylobacter* isolations were done by LSD. The Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario conducted the *Campylobacter* susceptibility testing.

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LIST OF ABBREVIATIONS

ABF	Antibiotic Free
AEI_G	Group Antimicrobial Exposure Incidence
AEI_P	Parenteral Antimicrobial Exposure Incidence
AFLB	Agri-Food Laboratories Branch
AMR	Antimicrobial Resistance
ACSSuT	ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline
AST	Antimicrobial Susceptibility Testing
AMU	Antimicrobial Use
ARIS	Automated Reading and Incubation System
BA	Blood Agar
Bp	Base pairs
BPW	Buffered peptone water
° C	Degrees Celsius
CI	Confidence Interval
CAHFSE	Collaboration in Animal Health and Food Safety Epidemiology
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standards Institute
CQA	Canadian Quality Assurance
DANMAP	Danish Program for Surveillance of Antimicrobial Resistance
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
GEE	Generalized Estimating Equations
ICC	Intra-Class Correlation
ICEPT	International Centre for Enteric Phage Typing
IMS	ImmunoMagnetic Separation
IQR	Inter-quartile Range
g	Grams
h	Hours
Kg	Kilograms
L	Litres
LIA	Lysine iron agar
m	Minutes
MIC	Minimum Inhibitory Concentration
mg	Milligrams
MHBA	Mueller-Hinton Blood Agar
ml	Milliliters
MRSV	Modified Semi-Solid Rappaport Vassiliadis

multiresistant	Resistant to two or more antimicrobials
N	Number
NARMS	National Antimicrobial Resistance Monitoring System
OIÉ	World Organisation for Animal Health (Office International des Épizooties)
OR	Odds Ratio
pansusceptible	Susceptible to all drugs tested
PCR	Polymerase Chain Reaction
PDS	Prairie Diagnostic Services
ppm	parts per million
PPS	Pooled Pen Sample
PQL-2	Second-Order Penalized Quasi-Likelihood
RAM	Rambach Agar
RCCT	Randomized Control Clinical Trial
RIGLS	Restricted Generalized Iterative Least-Squares
RT-PCR	Real-Time Polymerase Chain Reaction
RR	Risk ratio
RV	Rappaport Vassiliadis
s	second
S	Standard Deviation
SE	Standard Error
SAMS	Sensititre Automated Microbiology Systems
TS	Trypticase Soy
TSI	Triple Sugar Iron
TT	Tetrathionate
µl	Microlitre
VCP	Veterinary - Client - Patient
WHO	World Health Organization
XLT4	Xylose-Lysine-Tergitol 4 Agar

CHAPTER 1
INTRODUCTION

Antimicrobial resistant bacteria from livestock are a food safety hazard; bacteria can contaminate carcasses and persist through food-processing and handling to infect consumers (1-5). Antimicrobial use selects for resistant bacteria (6-15). When pigs receive antimicrobials, susceptible bacteria die. While it is desirable to kill pathogens, the loss of susceptible normal microbiota creates an ecological niche into which resistant bacteria can propagate and spread (16). In North America, pigs commonly receive antimicrobials through feed to treat disease, prevent disease and improve growth, and less commonly through water and by injection to control or treat infections (17-21). Laboratory experiments, controlled trials and observational studies have found antimicrobial exposure can select for antimicrobial resistance (10-13,22,23). However, most available descriptions of antimicrobial use in North American swine are qualitative (17-21,24,25). Quantitative data are needed to better understand the relationship between antimicrobial exposures and resistant bacteria in healthy pigs.

Twelve thousand cases of campylobacteriosis and six thousand cases of salmonellosis are reported in Canadians annually (26). These counts represent the 'tip-of-the-iceberg' as many cases go unrecognized and unreported (27). Antimicrobial resistant pathogens, including foodborne *Salmonella* and *Campylobacter*, cause greater morbidity than their susceptible counterparts; people taking antimicrobials are at increased risk of infection, and treatment failure and severe disease are more common with resistant infections (28-31). Pork is among the three most commonly consumed animal proteins in Canada (32). The rates of AMR in bacteria from

pigs at slaughter, and pork at retail, are intermediate between those reported in beef and chicken (33). So although the number of foodborne infections attributable to pork is unknown in Canada, there is undoubtedly some potential to decrease the burden of disease if on-farm interventions can minimize resistant meat contaminants. However, before measures to control AMR can be developed for swine farms, more data are needed describing the frequency and patterns of resistance in these zoonotic bacteria. These data will provide insight into the epidemiology of AMR on-farms, and will serve as a benchmark for future data comparisons. To date, the only published on-farm description of AMR in *Salmonella* from pigs on-farms described grow-finish pigs in Alberta (34). No on-farm studies are available describing AMR in *Campylobacter* from pigs.

Describing AMR in commensals provides insight into the selective pressure for resistance in gastrointestinal bacteria (3). Resistant commensal bacteria, such as *E. coli,* do not typically cause disease. Rather, these bacteria serve as a reservoir of antimicrobial resistance genes that can be transferred to other bacteria in the gut, including zoonotic pathogens such as *Salmonella* (3,35-37). Antimicrobial resistance is the ability for bacteria to survive in the presence of normally inhibitory drug concentrations (38). Most resistance phenotypes can arise through the expression of a variety of AMR genes (39-43). Because *E. coli* serves as a reservoir of resistance genes, describing the resistance genes documents the diversity of resistance determinants available for dissemination to other bacteria (42,44). These data can also distinguish between apparently equivalent resistance-phenotypes in different populations of animals and generate hypotheses about gene linkages and co-selection (39,40,42,43). Such findings provide insight into the evolution of AMR (44). Targeted research projects have

described AMR in *E. coli* from swine farms in Ontario and British Columbia, and the AMR genes carried by porcine *E. coli* in Ontario (23,40,42,45). To date, AMR in *E. coli* from western Canadian swine farms has not been described in the published literature

In response to growing concern about antimicrobial resistance, international authorities have recommended that countries monitor their agricultural antimicrobial use and AMR in bacteria from food animals (46-49). Canada has responded to these recommendations by conducting AMR surveillance in abattoirs and retail outlets (33). This pilot project was initiated in 2004, in part, to assist Canadian authorities in their efforts to develop the on-farm branch of their national surveillance program. The studies described in this thesis were part of a cross-sectional study conducted in a convenience sample of 20 Alberta and Saskatchewan swine farms. The main objective of this project was to describe the AMR of gram negative enteric bacteria from healthy pigs, and investigate the relationship between resistance and antimicrobial exposures in swine herds.

The initial chapter of this thesis provides a review of the pertinent literature (Chapter 2). Chapter 3 describes the antimicrobial use on study farms; these data were used in Chapters 7 and 8 to conduct antimicrobial resistance and exposure risk-factor analyses. Chapter 4 presents the frequencies and patterns of resistance in *Salmonella* isolates. The majority of this thesis was devoted to AMR in *E. coli*; phenotypic (Chapter 5) and genotypic (Chapter 6) AMR were described, along with the associations between on-farm antimicrobial use and resistance (Chapter 7). The final study describes the resistance of *Campylobacter* isolates (Chapter 8). In this chapter, antimicrobial use risk factors for resistance to the macrolides and quinolones are

described. The concluding chapter (Chapter 9) reviews and discusses the important findings of this thesis. It closes with identified research needs for future on-farm AMR studies.

Each chapter of this thesis was written as an independent paper. While this makes the study findings more usable, it inevitably causes repetition between chapters, particularly in the materials and methods. For those who read this thesis as a complete unit, I apologize for this inconvenience and am impressed with your perseverance.

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Antimicrobial resistant bacteria from livestock are a food safety concern. The incidence of disease from resistant foodborne bacteria is higher in people taking antimicrobials than in people without antimicrobial exposure. Resistant *Salmonella* and *Campylobacter* infections are difficult to treat because of limited antimicrobial selection and increased disease severity (1-4). Commensal bacteria, such as *Escherichia coli*, can transfer resistance genes to other commensal and pathogenic bacteria while passing through the human gastrointestinal tract (5-9). Thus, AMR in food animals is a public health problem because it increases foodborne disease incidence and severity and potentially provides resistance genes to human enteric bacteria.

Beef, chicken and pork are the most commonly consumed animal proteins in Canada, and pork is the most common worldwide (10,11). Antimicrobials are used extensively in pork production and resistant bacteria have been identified in pigs on-farm, at slaughter and in pork at retail (12-15). On-farm studies can evaluate antimicrobial use and management practices as risk factors for resistance in pigs (16,17). Ultimately, on-farm studies may identify interventions that minimize AMR in pigs and consequently foodborne disease in people.

2.2 Methods

This literature review discusses antimicrobial resistance (AMR) in *Salmonella* spp., *E. coli* and *Campylobacter* spp. from pigs on North America farms. The review begins by describing antimicrobial use in pigs. Following this, the public health significance of AMR and extent of resistance on swine farms is considered for each bacterium. Supporting and refuting evidence that antimicrobial use causes resistance is also investigated. The objective of this review is to document consistent findings in North America, consider reasons for conflicting findings, and identify areas requiring further research. Therefore, data were restricted to North American studies conducted since 1990 unless North American on-farm data were scarce or seminal studies occurred outside of these geographical and temporal restrictions.

Standard search engines including AGRICOLA, CAB abstracts, and Ovid MEDLINE® as well as non-scientific search engines including Google™ were used for literature searches. Search terms included, but were not limited to, combinations of the following: antimicrobial, antibiotic, use, exposure, treatment, susceptibility, resistance, *Campylobacter*, *Escherichia coli*, *Salmonella,* swine, pig(s), herd and farm. Cited references were examined for additional resources. An English language restriction was used.

2.3 Antimicrobial use in North American swine

There is worldwide concern about AMR in human medicine. Though most resistance in human pathogens is presumably due to antimicrobial use in humans,

veterinary drug use also contributes to this problem (9,18,19). International agencies, including the World Health Organization (WHO) and the World Organisation for Animal Health (OIÉ), recommend countries monitor the amounts of antimicrobials used in food animals (20,21). These data are needed to evaluate judicious-use campaigns, make recommendations for appropriate antimicrobial use, and conduct AMR risk assessments (22,23).

Numerous metrics for describing antimicrobial use have been proposed or used including total weight consumption, therapeutic costs, and treatment doses (24). In the last decade, many European Union (EU) countries have described their agricultural antimicrobial use by weight consumption (25-28). Denmark has collected farm-level data, while other countries have estimated national consumption from sales data (25-29). In contrast to these EU countries, Canada and the United States have not reported agricultural antimicrobial sales numbers. Reasons for not reporting this information include confidentiality concerns and an inability to stratify use by species, production class, or indication (23,30). Stratified exposure estimates are important for explaining changes over time. Total weight consumption estimates assume equivalent potency of antimicrobials. In reality, potency varies between products, which makes weight estimates of questionable value for comparing use between countries or describing the selective pressure for resistance (24,31). For these reasons, a standard metric that accounts for potency is desirable.

Human pharmaceutical consumption is described by the internationally accepted system of defined daily doses (DDD). The DDD reflects the average maintenance dose per day in a human adult for the drugs major indication. Comparing DDDs provides a rough estimate of consumption: it is not exact because the DDD may differ from the prescribed daily dose (PDD) (24,31). A veterinary classification based on the DDD concept has been implemented in Denmark (31). Animal daily doses (ADD) have been established which are specific to age-class and species (31). To date, the ADD system has not gained widespread international acceptance because of concerns about differences in prescribed doses (as primary indications differ among regions) and the variable size of animals within production classes. Substantial differences between the ADD and PDD limits the validity of extrapolating data to consumption estimates (31).

The ADD concept has been used in a peer-reviewed paper describing antimicrobial use in pigs. Timmerman et al. described group treatments in pigs in Belguim using ADD_{pigs} that reflected drug use in Belgium. Despite using a national metric, substantial differences were identified between the used daily dose (UDD) and PDD (32). Such within country discrepancies between ADD, PDD and UDD could plausibly be magnified at the international level. This is one factor that has hindered development of ADD_{animal} definitions. A second concern with using PDD-type data to describe antimicrobial use is that assumptions are often necessary. Timmerman et al. used a growth table to make assumptions about pig body weight to estimate consumption. Data assumptions should be clearly stated: in this instance, differences in growth rates and feed

intakes between herds could not be accounted for. The potential effect of this variability on antimicrobial use estimates was not addressed.

Antimicrobial use in North American pigs has been described through targeted on-farm studies (12,33-39). All studies stratified exposure by production phase and administration route, which makes them valuable in comparison to national weight consumption estimates. Although comparisons between studies are hindered by the variety of metrics used, each provided valuable insight into the reasons antimicrobials are used. Collectively, these studies provide insight into trends in the proportion of herds using drugs and commonly used products.

North American pigs are managed in phases. The suckling phase extends from birth until weaning. Pigs are weaned at an average of 19.3 days (Standard deviation (*S*), 0.2), although there is substantial variation among herds (40). Large herds tend to wean pigs earlier (average, 17.2 days) while small herds tend to delay weaning (average, 30.0 days) (40). Weaning marks the beginning of the nursery phase. Piglets may be moved to a separate pen in the same room as sows but are more commonly moved to separate rooms or even sites. The nursery phase is associated with increased disease risk because pig stressors, including mixing and diet changes, occur concurrently with declining maternal immunity (41). Pigs generally remain in the nursery phase until they are 6 to 10 weeks of age (average, 61.8 days; S, 0.6) (40). There is less variation between small and large herds in the timing for moving pigs to the grow-finish phase (40). The grow-finish

phase completes the production cycle. Pigs remain in this phase until marketed or selected for the breeding herd.

North American swine producers use antimicrobials to treat disease, prevent disease, and improve feed efficiency and daily gain (13,42). Antimicrobials are predominantly administered by feed; more than 90% of producers use feed-grade antimicrobials. Feed-grade antimicrobial use is more common in younger pigs. As pigs age, fewer diets contain medication (33,35,37,38). Recent reports indicate that products containing combinations of tetracyclines, sulfonamides, and penicillin are commonly used in nursery pigs, while tylosin is the most commonly used drug in grow-finish pigs (33,38). In-feed medication use appears to be relatively static over time within herds. Rajić et al. reported that most producers used antimicrobials in diets more than 95% of the time in the previous 12 months (38). Furthermore, Dunlop et al. found few producers changed medication practices over 18 months (43). This suggests that producers use feed-grade antimicrobials to manage disease risk rather than treat disease occurrences.

Antimicrobial use in water is less common than in feed. In Alberta, 50% of study herds used water medications in nursery pigs, 21% in grower pigs and 18% in finisher pigs (38). Similarly, 25% of surveyed Ontario producers and 30% of United States producers used water medication in grow-finish pigs (33,37). In Alberta, penicillin was the most common water soluble antimicrobial used in all production phases. Other commonly used products in grow-finish pigs included chlortetracycline and sulfamethoxine in the United States and dimetridazole and tetracycline in Alberta

(33,38). Dimetridizole was banned from use in pigs in Canada in 2003 (44). Two studies have found large herds are more likely to administer antimicrobials through water than small herds (33,37). In Canada, 7% of herds produce half of the nation's pigs (45). In western Canada, this trend is even more pronounced; 1% of herds produce half of Saskatchewan's pigs (46). Hence, accurate estimates of antimicrobial exposure require data on the proportion of herds using water soluble antimicrobials stratified by herd size. Failing to account for herd size could substantially underestimate antimicrobial exposure.

Parenteral antimicrobial use is more common in suckling piglets and sows than in nursery or grow-finish pigs (38,43). As pigs approach market, fewer herds use any injectable drugs and those continuing to use injectable antimicrobials report lower exposure rates (43). Pigs are exposed to substantially less antimicrobial (by volume) through injection than through feed or water because the duration of treatment is shorter and fewer animals are exposed. Despite this, it is important to describe injectable antimicrobial use because some antimicrobials are only available as injectable products.

Fluoroquinolones and third generation cephalosporins are among the drugs considered critically important in human medicine, hence the use of these drugs in livestock is of particular interest (47,48). There are currently no fluoroquinolones licensed for use in pigs in Canada (49). Ceftiofur is the only third generation cephalosporin licensed for use in pigs and is available only as an injectable drug (49). An Alberta study found ceftiofur was used in 10% percent of herds which made it the fourth most common injectable product in nursery pigs and the fifth most common product in

grower and finisher pigs(38). A United States report indicated ceftiofur was the third most commonly used injectable antimicrobial on swine farms (33). Extended-spectrum cephalosporin resistant *Salmonella* are a public health problem, and human cases have been occasionally linked to livestock sources (50). Furthermore, *Salmonella* and *E. coli* from cattle, pigs, and people carry the extended-spectrum beta-lactamase gene CMY-2 on similar plasmids (6,51). These findings suggest that using third generation cephalosporins in food animals may influence human exposure to extended-spectrum cephalosporin resistant pathogens. Such links between human and veterinary medicine reinforce the importance of describing injectable antimicrobial use in livestock.

The rate of antimicrobial exposure in North American pigs is essentially unknown. Rajić et al. categorized herds by the frequency of antimicrobial use in feed and water. Ninety percent of farrow-to-finish herds medicated nursery diets more than 95% of the time and 82% of herds medicated grower diets more than 95% of the time (38). However, this study did not report the duration of exposure. Pig-exposure incidence has only been reported for injectable drug use in Ontario herds. That study found highly variable exposure rates between phases and herds (43). Rate based data might enable estimation of antimicrobial consumption in Canadian pigs, potentially improving the precision of antimicrobial use risk factor analyses.

In summary, most swine herds in North America use antimicrobials and many use them extensively (33,37,38). Three studies found almost all herds use feed-grade antimicrobials and one-third to one-half use water-soluble drugs (33,37,38). These

findings were consistent despite different locations, times and data collection methods. More information is needed describing the rate of antimicrobial use within herds. These data would allow authorities to estimate national antimicrobial consumption and minimize fallacies when investigating the association between antimicrobial use and resistance in pigs.

2.4 Antimicrobial Resistance in *Salmonella* from pigs

Salmonella is the second leading cause of foodborne disease in Canada, surpassed only by *Campylobacter* (52). Six thousand cases of human salmonellosis are reported annually and for each reported illness an estimated 13 to 37 cases remain unreported (53). In Canada, over one-third of human clinical isolates are resistant to at least one antimicrobial, and 11% are resistant to five or more (12). This is concerning because resistant *Salmonella* can cause even greater morbidity than their susceptible counterparts. Treatment failure, increased disease severity, and increased risk of infection in people taking antimicrobials for other reasons all contribute to an increased disease burden (1,54-56). Antimicrobial resistant *Salmonella* in livestock are a food safety concern because most resistant *Salmonella* are acquired, rather developing resistance in people post-infection (14,57,58).

Although Canadian estimates are not available, 15% (95% Confidence interval (CI), 5 to 25%) of *Salmonella* cases in The Netherlands and 9% (95% CI, 8 to 10%) in Denmark have been attributed to pork consumption (59,60). In the United States, pork consumption is estimated to cause 99,430 cases (90% CI, 20,970 to 245,560) of salmonellosis annually (61). Globally, most human non-typhoid *Salmonella* infections

are *S.* Enteritidis (65%) followed by *S.* Typhimurium (17%) (62). In Canada, these serovars each account for approximately 20% of the reported human cases (52). *S.* Typhimurium (including variant Copenhagen) is also among the most common serovars reported in healthy pigs on-farm (39,63-67). The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) found that *S.* Typhimurium (including var. Copenhagen) accounted for 15% of isolates collected from healthy pigs at slaughter (12).

Antimicrobial resistance in *Salmonella* spp. from sick pigs has been described (12,51,68). Clinical isolates had more resistance and a different serovar distribution than *Salmonella* from healthy pigs at slaughter (12,68). This increased resistance could be due to any, or all of the following: antimicrobial use in sick pigs, associations between resistance and serovar, or associations between resistance and virulence genes (64,69,70). Monitoring AMR in *Salmonella* from sick animals provides a sensitive warning for emerging resistance but does not reflect the consumer risk from pork. For this, *Salmonella* must be isolated from healthy animals that enter the food chain. *Salmonella* from retail meats more closely reflects the consumer risk from improperly prepared food, while isolates from pigs in abattoirs reflect the risk of carcass contamination. *Salmonella* from pigs on farms provide a baseline prevalence of resistance entering the food processing chain from live pigs (71).

Resistance in *Salmonella* collected at slaughter can differ from isolates collected on-farm. Nollet et al. reported significantly less resistance in *Salmonella* spp. isolated

from the mesenteric lymph nodes, jejunum and colon than from fecal samples collected on-farm (72). Erdman et al. found additional serovars and resistance patterns at slaughter that had not been identified on-farm (73). Gebreyes et al. found the genetic fingerprints of *S.* Typhimurium (including var. Copenhagen) collected at slaughter were different from those on-farms, but identical to those collected from transport trucks. This indicates that pigs can be infected during transport, even when trailers are washed between loads (64). Infection during transport and lairage means antimicrobial use and farm management practices should be evaluated as risk factors for resistance in *Salmonella* collected on-farm. Additionally, post-shipping infection means that abattoir-based and on-farm studies are complementary but not equivalent. Abattoir studies can sample pigs from many herds; samples can be collected proportional to regional or national production and better reflect the consumer risk from resistant bacteria. In contrast, on-farm studies can investigate risk factors for the development and persistence of resistance in pigs.

In Canada, most *Salmonella* AMR data are from abattoirs or clinical isolates (12,68,70,74,75). On-farm data are available from healthy grow-finish pigs in Alberta and national monitoring has been initiated but results are not yet available (12,76). In the United States, data from targeted research projects and national monitoring are available (39,63-65,77). The proportion of isolates susceptible to all drugs on tested (pansusceptible) was higher in Alberta (53%) than in the United States (6%) when similar panels were used (39,76). Rajić et al. and CAHFSE also reported the same top three drug resistances but CAHFSE had markedly more resistance: tetracycline, 39 vs. 90%; streptomycin, 26 vs. 69% and sulfamethoxazole, 21 vs. 63% (39,76). Initially, it seems

plausible that Rajić et al.'s geographic limitation to Alberta could account for this difference. However, the Alberta data were similar to the rates of resistance reported by CIPARS in *Salmonella* from healthy pigs at slaughter; tetracycline 42%, streptomycin 26% and sulfamethoxazole 28% (12). Therefore, other factors should be investigated to explain these substantial differences between *Salmonella* from Canadian and American swine farms. Such factors could include serovar differences, management practices, or antimicrobial exposures.

Resistance to extended-spectrum cephalosporins and fluoroquinolones among *Salmonella* is particularly important because these antimicrobials are used to treat salmonellosis in people (48,57). To date, North American on-farm studies have not found *Salmonella* resistant to ciprofloxacin (39,63-65,76). Likewise, no resistance has been identified to ceftiofur or ceftriaxone in abattoir or on-farm studies in Canada (12,76). This is markedly different from United States farms where 21% of *Salmonella* were ceftiofur resistant (39).

A recent investigation describing *Salmonella* AMR in three herds found significantly less resistance in sows than in production animals (72). *Salmonella* serovars can differ between production phases within herds (78). This, along with antimicrobial exposure and physiological differences, may explain why antimicrobial resistance differs between production phases (38,43). Currently, data describing AMR in *Salmonella* from pigs distant from market are scarce. These data might provide insight into how resistance

persists and spreads within herds, and identify phase specific risk factors. Defining risk factors for resistance is crucial for developing AMR intervention plans.

Studies considering the effect of antimicrobial exposure on resistance in *Salmonella* have reached contradictory conclusions. Two studies found antimicrobial exposure in feed had no or minimal effect on resistance in experimentally induced *S.* Typhimurium (79,80). Resistance to 12 drugs did not differ between *Salmonella* from pigs exposed to 100 ppm of tylosin for 56 days (N = 10 pigs; n = 107 *Salmonella*) and *Salmonella* from pigs fed a non-medicated control ration (N = 10 pigs; n = 110 *Salmonella*) (81). Similarly, no significant time-treatment-antibiotic interactions were found in a study comparing three antimicrobial exposures and a non-medicated control (n = 12 pigs per group) indicating resistance did not vary over time within treatments. In contrast, an observational study found *Salmonella* spp. from antibiotic free (ABF) herds were significantly more likely to be pansusceptible than isolates from conventional swine herds (82). The odds of resistance to every antimicrobial except tetracycline were higher in *Salmonella* from the conventional herds compared to ABF herds (82). The difference between these experimental and observational studies could be attributable to serovar because resistance is associated with serovar (64,70). The experiments only used *S.* Typhimurium while the observational study did not describe the serovars. The differences may also be due to the antimicrobial exposures tested; *Salmonella* resistance may develop differently with certain exposures (such as drug, dose, duration or frequency) and the experimental and on-farm exposures could have differed substantially. Conversely, the on-farm study was cross-sectional and cannot ascribe causality (83). Conventional and

ABF herds could have had *Salmonella* with different resistance patterns because of variables other than antimicrobial use. Management variables were not considered as independent risk factors or potential confounders (82).

Antimicrobial resistant *Salmonella* are a food safety hazard given that resistant salmonellosis outbreaks have been traced to pork products (84,85). However, monitoring AMR in *Salmonella* is challenging because sub-clinically infected pigs shed intermittently, barns fluctuate between a *Salmonella*-positive and apparent-negative status, and isolation techniques can have poor sensitivity (67,86). Two studies recently illustrated the challenges of studying AMR in *Salmonella*. The first was a trial examining AMR in pigs with and without subtherapeutic chlortetracycline exposure in feed. Twenty-two barns with a history of *Salmonella* spp. contamination participated. Despite this, *Salmonella* spp. were isolated from only 15 of 2112 fecal samples (17). A second study compared AMR in *Salmonella* spp. from ABF and conventional swine herds. Four of the seven herds had no positive samples isolated. Additionally, the 143 isolates collected may have represented as few as 44 unique strains because multiple isolates were harvested from samples cultured with selective enrichment (87).

In contrast to *Salmonella*, *E. coli* are highly prevalent and easily isolated. *Salmonella* and *E. coli* from pigs can share resistance genes in vitro and molecular evidence suggests transmission occurs in vivo (6,88). The resistance phenotypes for these bacteria ranked in a similar order in both the CIPARS and CAHFSE reports (12,39). To date, the feasibility of using *E. coli* as a herd level sentinel for AMR in *Salmonella* has

not been reported. As a sentinel species, *E. coli* would provide more cost effective monitoring of resistance and might improve the power of investigations for resistance risk factors.

In summary, *Salmonella* in North American pigs carry a concerning level of resistance (12,39,76). The potential food-safety risk from resistance to quinolones and extended-spectrum cephalosporins should impel more on-farm studies (57). As the prevalence of resistance becomes better known, and associations with serovar become better described, risk factors for resistance can be investigated. Ultimately, the goal of on-farm studies should be to identify interventions that control or reduce antimicrobial resistant *Salmonella* in pigs. Farm-level sentinel bacterial such as *E. coli*, have the potential to advance this objective and should be investigated.

2.5 Antimicrobial Resistance in commensal *Escherichia coli* from pigs

2.5.1 Phenotypic antimicrobial resistance

Antimicrobial resistance in *E. coli* poses at least three health concerns for humans and animals. Two concerns are only relevant to pathogenic *E. coli*; resistant infections have an increased risk of treatment failure and are potentially more virulent than their susceptible counterparts (89-93). The third concern applies to both pathogenic and non-pathogenic *E. coli*; antimicrobial resistant bacteria have a competitive advantage in people or animals taking antimicrobials as they are more likely to colonize and persist in the gut (56,93). Colonization with resistant commensal strains is a concern because *E. coli* can transmit resistance genes to other bacteria. Resistance gene exchange can occur between bacteria from diverse ecological niches, including from animals to humans (6-8).

Therefore, AMR in *E. coli* is a public health concern, although the magnitude of risk is undefined.

Unless otherwise stated, this review describes commensal *E. coli* from healthy pigs. This is noteworthy because the rates of phenotypic resistance and the AMR genes differ between porcine pathogenic and commensal *E. coli* (90,92). Studying commensal *E. coli* is important because they reflect the reservoir of resistance genes available for dissemination to other bacteria, and indicate the selective pressure for resistance in the normal gut flora (9,71). Because *E. coli* are prevalent in healthy animals, they enable resistance to be compared between pigs in different locations and times, and between pigs and other species (9,94).

Antimicrobial resistance in *E. coli* is studied on-farm because transportation, stress and lairage can alter the rates and patterns of resistance in pigs (95-98). In Canada, on-farm studies have been conducted in British Columbia and Ontario and national monitoring was initiated in 2006 (12,15,99). In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) collected *E. coli* from swine farms in 2000 and CAHFSE collected repeated samples from 2003 to 2005 (39,77,100). Research projects have also described resistance in sows and younger pigs in Tennessee (101,102). With the exception of the national surveillance projects, study herds have been selected based on antimicrobial use.

Observed antimicrobial resistance rates, and the most common drug resistances, have been similar in Canada and the United States. In the United States, resistance was most common to tetracycline (86 and 92%), sulfamethoxazole (42 and 44%) and streptomycin (29 and 35%). A low rate of resistance to ceftiofur (1.5 and 3.2%) and no resistance to ceftriaxone or ciprofloxacin was reported (39,77). Dunlop et al. described resistance in *E. coli* from pigs in Ontario while Akwar described *E. coli* from pigs in both Ontario and British Columbia (15,99). These studies also found *E. coli* were most commonly resistant to tetracycline (71 and 81%) and sulfamethoxazole / sulfisoxazole (38 and 59%) (15,99). Akwar also found frequent resistance to streptomycin (32%), no resistance to ceftiofur or ciprofloxacin, and 0.3% resistance to ceftriaxone (99).

The studies describing *E. coli* AMR on Ontario and British Columbia swine farms were cross-sectional (99,103). Both studies purposively selected herds by antimicrobial use and, therefore, extrapolating the reported resistance prevalences to other herds in these provinces may be inappropriate (99,103). Of these two studies, the external validity of the study by Dunlop et al. was better because the resistance prevalences were standardized to reflect Ontario hog farms. The author used a mail-based survey to describe the drug-use in Ontario herds. This knowledge of the sampling frame was used to weight the prevalence of resistance in each drug-use strata by the percentage of Ontario herds in each stratum (103). In contrast, Akwar's findings could have underestimated the prevalence of resistance in Ontario and British Columbia. Farms using in-feed medication had significantly more resistance than farms without exposure and the proportion of study herds with no feed-grade drug use was higher than the source

population (37,99). However, it is also important to consider that Akwar's findings may overestimate resistance in finishing pigs. *Escherichia coli* from weaned pigs had significantly more resistance than from finisher pigs, but resistance to individual drugs was pooled across production phases (99). This could be important if these data were compared to *E. coli* from close-to-market animals. Preliminary results describing on-farm *E. coli* AMR in Alberta have been published (104). Complete findings from Alberta, along with the findings of this thesis, may identify differences between herds in eastern and western Canada as well as changes over time within Alberta herds (104).

On-farm studies have investigated the variation in resistance at different organizational levels (17,105,106). Focusing limited resources on the most variable levels improves the chance of finding risk factors for resistance (83). Three studies have provided information on how resistance clustered within pigs, pens and production companies (17,105,106). Brun et al. explored variation at two hierarchical levels; the variation in repeatedly sampling animals as they moved through a production phase, and the variation in isolates from an animal within a sampling (106). The variation in resistance between sampling times within a production phase was assessed by sampling sows three weeks post-farrowing, at weaning, and four weeks before the next farrowing. Offspring from these sows were sampled at three weeks of age, upon entry to the grower barn and pre-slaughter. The variation in resistance between *E. coli* within an animal at each sampling was explored by testing 10 isolates per sample. Isolates were tested for susceptibility to 11 antimicrobials. Every resistance outcome, except ampicillin in sows, had negligible variation within an animal relative to the variation within a production

phase. This suggests testing many isolates per animal is not valuable. It also indicates that resistance in a pig class (i.e. sows) cannot be described unless all points in the production cycle (i.e. breeding, gestation, farrowing) have been sampled. Again, isolates should represent as many animals as possible. Brun et al.'s study sampled only two farms. Although variation between herds could not be described, herd was a significant fixed effect for resistance to some drugs. This suggests that variation between herds might be important for describing resistance in a target population (106).

A second study described the variation in resistance within a barn of finishing pigs (105). More than 80% of the variation was attributed to differences between pigs within pens, compared to pens within rooms, and rooms within the barn. Variation between isolates within an animal was not considered (105). Dunlop et al.'s findings suggest risk factor studies should target individual animals rather than pens or rooms. However, growing pigs are managed in groups. As it may not be feasible to implement pig-level interventions, it may be more valuable to describe resistance at the level of the grow-finish phase. Simulation modeling determined phase level resistance was optimally described by pooling feces from 20 animals and selecting five isolates per pooled sample. This sampling plan was used in the subsequent on-farm study. Substantial variation in resistance occurred between herds with different antimicrobial exposure histories (15). For each resistance, antimicrobial use risk factors were identified, while other herd level variables were not important confounders (16). This work suggests the production phase is a rewarding level to investigate AMR risk factors (15,16,105).

A third study compared AMR in *E. coli* from finisher pigs exposed to diets with, and without, subtherapeutic chlortetracycline (17). Three production companies each enrolled two to twelve finishing barns in the trial. Treatment allocation was at the finishing barn level. Variation was considered at the isolate, pig and production company level while variation between barns was not investigated. Consistent with previous reports, most variation occurred at the pig level, although more variation was found between isolates within a pig than reported by Brun et al. (105,106). Based on this, Funk et al. suggest risk factor studies should target more pigs per farm (production company), and more *E. coli* per pig, rather than more farms (17). This conclusion is appropriate for antimicrobial exposure trials; however, observational studies describing associations between herd- or phase-level antimicrobial exposure and resistance still require many herds because variable exposures are needed to test hypotheses.

Together, these three studies provide guidelines for investigating AMR on swine farms. First, antimicrobial use is a risk factor for AMR in pigs (16,17). Therefore, herds purposively selected for antimicrobial use, although ideal for risk factor studies, may provide biased estimates of resistance in a population. Second, if findings are inferred to the entire herd, sampling should be stratified across production phases. The results obtained in one production phase are not inferable to another (106). Third, the optimal sampling plan differs with the study objective. Studies describing the prevalence of resistance in a region, or investigating phase level risk factors, should collect composite samples from many herds. Studies investigating pig- or pen- level risk factors should collect samples from as many individual animals as feasible with a limited number of

isolates per sample. However, it may not be rewarding to investigate pen-level risk factors because within pen variation is minimal. Pig-level risk factors are more likely to be identified but may not result in practical interventions (105).

2.5.2 Genotypic antimicrobial resistance

Each resistance phenotype can arise from the expression of numerous different resistance genes (90,107). Thus, AMR genes can identify differences masked by phenotypic AMR. Describing AMR genotypes also provides insight into how resistance is transmitted, how genes interact and the potential for co-selection (108).

Genotypic AMR data are important for developing hypotheses on where resistance develops and how it spreads between animal and human populations. For example, *sul3* has emerged in human and pig populations in Europe, Canada and the United States (90,109-113). This suggests resistance is transmitted between these species and countries, although it does not indicate the original source of this gene (108). Although molecular epidemiology is in its infancy, this is an indication of how links between AMR in food animals and people may be explained.

Many genes encode for each resistance but a few genes often dominate in a bacterial population. For example, at least 36 genes encode for tetracycline resistance but three genes almost completely describe tetracycline resistance in *E. coli* from pigs: *tetA*, *tetB* and *tetC* (90,107,114-116). The relative distribution of these genes varies across swine populations (90,107,114-116). This variation could be due to temporal, antimicrobial exposure, or serovar differences. Maynard et al. noted temporal changes in

pathogenic *E. coli* collected over 23 years. From 1978 to 1994, few isolates carried *tetA*, and none carried *tetC*. In contrast, these genes each accounted for one-third of the tetracycline resistance from 1995 to 2000. Antimicrobial exposures or management systems may also select for different genes. Blake et al. found tetracycline resistant *E. coli* from extensively raised pigs did not carry *tetB,* while those from intensively raised pigs did not carry *tetA* (117).

As with *Salmonella*, some *E. coli* strains appear to have a predilection for certain AMR genes. Continuing with tetracycline resistance as an example, Boerlin et al. found *tetA* predominated in Enterotoxigenic *E. coli* (ETEC) while *tetB* was more common in pathogenic non-ETEC and in commensal *E. coli* (90). The association between *E. coli* type and chloramphenicol resistance genes has been studied more thoroughly. *Escherichia coli* from piglets with diarrhea in Oklahoma almost exclusively carried *cmlA* as did non-ETEC from pigs with diarrhea in Ontario (92,109). In contrast, chloramphenicol resistant ETEC from pigs in Ontario predominantly carried *catA1* and commensal *E. coli* carried *floR* (92). Examining associations between *E. coli* strains, virulence genes and AMR genes may clarify how AMR spreads and persists in pig populations. These associations should be accounted for when considering associations between risk factors such as antimicrobial use and AMR.

Antimicrobial resistance can be acquired through genetic mutation or by obtaining resistance genes (118). Once acquired, resistance genes are passed to successive generations of bacteria during replication. Bacteria can acquire resistance genes

independent of replication by taking up naked DNA (known as transformation), through bacteriophages (known as transduction), or through horizontal transmission of genetically mobile elements including plasmids, transposons and integrons (118). Horizontal transmission is the principal route for AMR dissemination (118). Within gram-negative bacteria, transmission by genetically mobile elements predominates. These elements link unrelated resistance genes, transmit them as a unit, creating multiple drug resistant bacteria. Excellent reviews provide detailed explanations of resistance gene transmission (119-121). For the purposes of this review, it is important to understand that multiple drug resistant bacteria have a competitive advantage over susceptible bacteria in the presence of any antimicrobial in their resistance phenotype (5).

Resistance genes are not linked arbitrarily. Certain genes have an affinity for each other; *sul1* and *aadA* genes often occur together because *sul1* is a component of type1 integrons and *aadA* is a common gene cassette accumulated by integrons (119,122). Other genes, such as *tetA* and *tetB,* are carried on incompatible plasmids (90). Studies using molecular epidemiological techniques, such as conjugation, DNA sequencing and hybridization, have provided evidence of gene linkages and transfers (8,92,123). However, because of laboratory time and expense most studies examine only a few purposively selected isolates. Therefore, results rarely provide definitive information on how genes behave in a larger bacterial population. Statistical associations provide an alternative for investigating gene interactions. Associations describe the probability of identifying genes together (90,92,107). Statistically, investigators can consider many more isolates and genes than is feasible in molecular experiments. Identified associations

generate hypotheses about how genes are transmitted, which can be tested with more expensive laboratory techniques.

Three Canadian studies have described the associations between AMR genes. One considered the association between AMR genes in *E. coli* O149:K91 obtained from pigs with diarrhea in Quebec between 1978 and 2000 (107). Two other studies considered the same set of *E. coli* from Ontario and are described together (90,92). Approximately half of these *E. coli* were from pigs with diarrhea while half were commensal *E. coli* from healthy finisher pigs. Nine genes were tested in both studies but only three corresponding associations were identified; *aac(3)-IV* / *catI* were positively associated while *sul1* / *sul2* and *tetA* / *tetB* were negatively associated. Four associations were identified only in Quebec isolates and ten were found in only the Ontario isolates. One association was observed in both studies but in opposite directions. In Ontario, *sul1* and *tetB* were negatively associated while in Quebec they were positively associated. Interestingly, *sul1* was positively associated with *tetA* in Ontario and in the Quebec isolates obtained from 1990 to 2000. This association was negative in the full set of Quebec isolates (1978 to 2000), which suggests associations between genes can change over time. In the Ontario isolates, the AMR genes were distributed differently between ETEC, non-ETEC pathogens and commensal *E. coli* (90,92). Certain virulence and resistance genes were also associated (90,92). Investigating only *E. coli* O149:K91 in Quebec, compared to diverse *E. coli* isolates in Ontario, could explain the different associations reported by these studies. However, temporal and geographic differences may also have been factors.

Through transformation, Travis et al. confirmed that many of the statistical associations were explained by plasmids carrying linked genes (92). Other associations occurred because clones, carrying multiple plasmids, had been tested. This emphasizes that statistical associations between genes only provide hypotheses about linkages, which require confirmation. It also illustrates the importance of selecting unrelated isolates because statistical associations assume independence (83).

Molecular epidemiology is a rapidly expanding area. Understanding the epidemiology of AMR genes will undoubtedly improve our ability to track the dissemination of resistance within bacterial population and between animals and humans (108). Few studies have investigated associations between antimicrobial use and AMR genes (117). As new technologies, automation, and decreasing costs make genotyping more feasible, future studies may investigate risk factors for resistance genes rather than resistance phenotypes.

2.5.3 Associations between antimicrobial use and resistance

Laboratory experiments, on-farm trials and observational studies have explored the effect of antimicrobial exposure on resistance in *E. coli* from pigs. In contrast to *Salmonella*, antimicrobial use has been associated with increased resistance in both observational and experimental studies (16,87,99,124-128). Laboratory experiments have provided causal evidence that antimicrobial exposure can directly select for resistance. In one study, nursery pigs receiving therapeutic doses (15 mg/kg) of tetracycline in feed for seven days had more tetracycline resistant *E. coli* than those receiving a subtherapeutic dose (1.5 mg/kg). Both treatment groups had more resistance than untreated controls

(126). In another study, weaned pigs exposed to apramycin continuously for 14 days had more apramycin resistance than pigs fed diets pulsed with apramycin (sequentially in three days and out three days) (125). These studies showed the dose and frequency of exposure to antimicrobials can affect AMR in pigs.

Co-selection occurs when antimicrobial exposure selects for unrelated drug resistances (5,129). Tetracycline exposure has selected for apramycin resistance experimentally. Young pigs, treated with apramycin, were exposed to different stresses including cold, crowding, and oxytetracycline. Pigs receiving oxytetracycline in feed at 100 g/ton for 141 days had more apramycin resistant *E. coli* than pigs with no antimicrobial exposure, and more resistant *E. coli* than pigs receiving apramycin but not oxytetracycline (130). This difference was significant between the second and ninth week of the trial. In another experiment, sows were allocated diets with or without oxytetracycline (100 g/ton for 14 days pre-farrowing). Piglets nursing treated sows had more apramycin resistant *E. coli* than piglets from untreated sows (124). These experiments show that antimicrobial use causes resistance to unrelated drugs in *E. coli* from pigs. Despite this, the clinical importance of these trials is difficult to interpret because treated and control animals had similar rates of resistant *E. coli* at the end of both studies despite isolation between treatment groups.

Experimental studies are the gold standard for determining causality (83). However, extrapolating conclusions from experimental studies to commercial swine operations may be invalid if environmental or animal factors differ substantially. To

address this weakness, other study methodologies have been used to investigate the association between antimicrobial use and resistance.

Randomized controlled clinical trials (RCCT) provide insight into how antimicrobial exposure can affect AMR in pigs on-farm. Management practices, such as stocking densities, feeding regimes and animal mixing, are similar to commercial barns. This improves the external validity of findings (83). An on-farm trial compared AMR in *E. coli* from finisher pigs receiving oxytetracycline (50 g/ton) for the entire grow-finish period (14 weeks) to *E. coli* from pigs without treatment. Treatment was allocated by barn after stratification by production company. Resistance to four antimicrobials was investigated. The odds of resistance to any drug were eight times higher in treated pigs compared to controls, and the odds of resistance to tetracycline, ampicillin and ceftiofur were each significantly higher in exposed pigs. Gentamicin resistance did not differ significantly between groups. These findings suggests low-dose, continuous exposure to tetracycline directly selected for tetracycline resistance and co-selected for resistance to unrelated drugs. This study's primary weakness was non-random treatment allocation. Randomization minimizes bias created by known and unknown risk factors so the extent to which this study's results are biased is difficult to determine (83). Until these results are substantiated, the possibility of bias should be considered when inferring these findings to other herds.

Of field epidemiological studies, RCCTs provide the strongest evidence for causation (83). However, they can only examine limited drugs, doses, and exposure

frequencies, all of which may affect resistance. Observational studies are ideal for investigating complex problems with many causes or hypotheses (83). To date, most on-farm studies have been cross-sectional. These have described associations between antimicrobial exposure and resistance at the national, herd and production phase level (16,87,99,128).

The Danish Program for Surveillance of Antimicrobial Resistance (DANMAP) collects *E. coli* from healthy animals at slaughter and sick animals (128). It also monitors national antimicrobial use in livestock. Monitoring programs can identify trends for retrospective investigation. The DANMAP program noted increased apramycin and gentamicin resistance following authorization of oral apramycin use in livestock. The odds of *E. coli* resistance to apramycin increased 1.9 times for every year following approval, and increased by 1.6 times with every 50U increase in apramycin consumption annually (128). The association between apramycin use and resistance in swine herds was investigated to avoid potential fallacies from inferring national-level associations to farms. No herds used apramycin in finisher pigs, but herds with apramycin and gentamicin resistant *E. coli* were significantly more likely to have used apramycin in nursery pigs. By considering associations between antimicrobial use and resistance at the farm and national level, this study identified risk factors for resistance and gave perspective to the public health risk from resistance in food animals.

Cross-sectional studies have compared AMR in *E. coli* from herds with different antimicrobial exposures. Mathew et al. compared the resistance in three herds with no

antimicrobial use to four herds using antimicrobials (87). Antimicrobial use was described in each phase but was only considered as the dichotomous variable 'no use' versus 'any use' in the analysis. State was the only herd descriptor. Dunlop et al. described associations between antimicrobial use and resistance in Ontario swine farms (16,103). Hog farms in Ontario were stratified by size and a subset of producers was surveyed about antimicrobial use. Respondents that met the inclusion criteria were eligible to participate. Thirty-four herds, selected for antimicrobial use practices, were enrolled. Antimicrobial use data were collected for two months, described by phase and product, and considered as risk factors for numerous resistances in *E. coli* from finisher pigs. Analyses adjusted for clustering within herds and considered numerous herd characteristics as potential confounders. Akwar expanded on this study by including more herds in Ontario, herds in British Columbia, and *E. coli* from weaned pigs (99). All three studies found that antimicrobial use was associated with increased resistance (16,87,99).

Of these studies, Mathew et al.'s provided the weakest evidence linking antimicrobial use to resistance (87). Based on a tendency for lower MICs in *E. coli* from herds without antimicrobial use the authors concluded, "excluding antibiotics from swine herds reduced the number of resistant bacteria…". This conclusion is flawed because cross sectional studies cannot ascribe causality (83,87). Simultaneous data collection on exposures and outcomes precludes stating which came first. This can create the problem of reverse causality. For example, a herd could have high levels of AMR, leading to increased disease, causing increased antimicrobial use. This study's second weakness was its failure to consider different antimicrobial exposures as risk factors, and herd variables

as confounders. This omission was likely because the only seven herds participated in the project. However, it resulted in the authors attributing the entire difference between herds to the antimicrobial exposure. Finally, this study provided no insight into high risk antimicrobial use practices.

In contrast, Dunlop et al. and Akwar both considered drug specific antimicrobial exposures in each production phase and considered management variables as potential confounders. Their findings corroborate other results from on-farm and laboratory experiments (16,99). Antimicrobial exposure in suckling piglets, nursery pigs and sows were each associated with at least one resistance outcome in *E. coli* from finisher pigs (16,99). This corresponds with experimental evidence that piglets from sows exposed to oxytetracycline in gestation diets had increased resistance (124). It also complements Jensen et al.'s observation that apramycin use in nursery pigs was associated with resistant *E. coli* in market age pigs in Denmark (128). Both Akwar and Dunlop et al. identified associations between antimicrobial use and resistance to unrelated drugs (16,99). Some associations have since been identified by others. For example, Dunlop et al. found the odds of ampicillin resistance were two times higher in herds using tetracyclines in grow-finish diets than those with no exposure (16). This is similar to Funk et al.'s observation that the odds of ampicillin resistance were 1.4 times higher in the herds allocated tetracycline exposure in finishing diets (17). Dunlop et al. also found tetracycline use in grow-finish diets was a risk factor for sulfisoxazole resistance (16). A corresponding association was subsequently identified between *tetA* and *sul1* in porcine *E. coli* from Ontario (90). These findings demonstrate the utility of cross sectional

studies; statistical associations can provide hypotheses for experiments and stimulate investigations into the physical linkage of resistance genes.

In conclusion, *E. coli* from pigs on farms in North American are commonly resistant to many different drugs (39,77,99). Antimicrobial use is a primary cause of this resistance (16,17,124). Further research is needed to identify what antimicrobial exposures pose the greatest risk for creating resistance to drugs important for human therapy (47,48,131). Molecular epidemiology will help on-farm studies identify interventions by distinguishing between apparently equivalent resistances and accounting for co-selection. In the meantime, producers and veterinarians should feel compelled to use antimicrobials as sparingly as possible.

2.6 Antimicrobial Resistance in *Campylobacter* from pigs

Campylobacter is the leading reported cause of bacterial foodborne enteric infections in many developed nations (132). In Canada, approximately 12,000 cases are reported annually with an estimated 23 to 29 unreported illnesses for every reported case (52,53). *Campylobacter* infections do not generally require antimicrobial therapy. However, macrolides may be prescribed for severe cases or immuno-compromised patients. *Campylobacter* infections are also treated with fluoroquinolones because ciprofloxacin is a front-line drug for undifferentiated gastroenteritis. However, resistance to fluoroquinolones is increasing, as are concerns about treatment failure (133-135). Similar to the situation with *Salmonella*, resistant *Campylobacter* infections may have increased virulence. People with ciprofloxacin resistant *Campylobacter* suffer from diarrhea longer than those with susceptible infections (3,4,136). Resistant *Campylobacter*

are generally acquired rather than selected for by antimicrobial use during therapy (4,135). This raises concerns about AMR in *Campylobacter* from food animals as most cases of *Campylobacter* are foodborne (134,137).

Eighty to ninety percent of campylobacteriosis cases are caused by *Campylobacter jejuni* while *Campylobacter coli* accounts for 5 to 10% (52,138). Although less significant than *C. jejuni*, *C. coli* can rank among the top four causes of enteric infection in people (139). *Campylobacter* infections in people are generally sporadic. This, along with the ubiquity of *Campylobacter* in many food animals, and the lack of an effective typing system, has hindered understanding the risk factors for infection (140).

Poultry are the primary reservoir of *C. jejuni* while pigs are a reservoir for *C. coli* (133,141,142). Almost all swine herds are *Campylobacter* positive and most pigs carry *Campylobacter* at slaughter (70 to 100%) (39,142,143). The importance of pigs and pork as a source of human campylobacteriosis is unclear (144-146). Many risk factor studies aggregate *C. jejuni* and *C. coli* cases (144). Because *C. coli* accounts for a small proportion of cases, risk factors unique to *C. coli* are likely to be missed (147). This may explain the discrepant findings of pork as a risk factor for *C. coli* infection (144-146).

A Canadian study investigated the relationship between *Campylobacter* from healthy pigs and ill people (148). Geographically and temporally related isolates had low relatedness. However, the clinical relevance of this null finding was difficult to interpret

because all species of *Campylobacter* were included (148). A case-case study conducted in England and Wales compared *C. jejuni* (controls) to *C. coli* (cases). A significant risk factor for *C. coli* infection was eating paté, which is commonly made with pork liver (147). This suggests that pork may play a role in *C. coli* infections in people. In contrast, a recent molecular epidemiological investigation found *C. coli* from ill people were closely related to isolates from poultry, including turkeys and ducks, but not to isolates from pigs (140). While risk factors for *C. coli* infection require more investigation, AMR in *Campylobacter* from pigs warrants investigation because macrolide resistance is highly prevalent and quinolone resistance is regularly observed (149-151).

No published studies have described AMR in *Campylobacter* from pigs on Canadian farms. A few have been conduced in the United States (100,152,153). The annual reports released by CAHFSE in 2003 – 2004 and 2004 – 2005 described *Campylobacter* AMR in nationally representative herds (39,100). In 2004 – 2005, broth microdilution susceptibility testing was used rather than the Etest®. Results were markedly different between years thus both reports are discussed. Knowledge of *Campylobacter* AMR in Canadian pigs is limited to abattoir based studies from Ontario and Quebec (149,151).

Although not every drug was tested in every study, *Campylobacter* spp. were most commonly resistant to tetracycline (range, 35 to 83%), erythromycin (28 to 77%), azithromycin (28 to 67%) and clindamycin (0 to 59%) (39,100,149,151-153). For these antimicrobials, the lowest resistance was reported by CAHFSE in 2004 – 2005. The

opposite situation occurred with chloramphenicol resistance; all studies reported 3% or less resistance except CAHFSE (2004 – 2005) which reported 24%. Of the above studies, CAHFSE (2004 – 2005) was the only study using broth microdilution for susceptibility testing. It was also the only report that did not provide the breakpoints used (39). These factors could account for the difference from other reports and the previous CAHFSE report (100,149,151-153). In all reports, resistance to nalidixic acid ranged from three to eleven percent. Ciprofloxacin resistance was minimal (<3%) with the exception of *C. coli* from Quebec pigs (11% resistance) (39,100,149,151-153).

In the United States, AMR in *Campylobacter* spp. from pigs raised in conventional and ABF herds has been described (153). Less than 10% of the isolates were resistant to chloramphenicol, ciprofloxacin, gentamicin and nalidixic acid, and resistance was not significantly different between herd types. In contrast, over half of the isolates were resistant to tetracycline and erythromycin and these resistances were more common in conventional herds. Conventionally raised pigs received oxytetracycline and tylosin in nursery and finisher feed, which suggests antimicrobial exposure increases certain *Campylobacter* resistances (153). Environmental factors also appear important in *Campylobacter* resistance. In ABF herds, tetracycline and erythromycin resistance was significantly higher in pigs raised indoors compared to outdoors. As in the previous study, chloramphenicol, ciprofloxacin, gentamicin and nalidixic acid resistance was less common and not associated with herd type (152). Management factors, including housing and feeding practices, appear important in the epidemiology of *Campylobacter* AMR (151-154).

There is a temporal correlation at the ecological level between the approval of fluoroquinolones for use in livestock and emergence of resistant *Campylobacter* in animals and people (133,134). Biologically it is plausible that fluoroquinolone use contributed to resistance in porcine *Campylobacter*. This is supported by experimental evidence; pigs exposed to enrofloxacin for five days had more resistant *C. coli* than controls and resistance persisted for 35 days post-exposure (155). However, risk factors other than fluoroquinolone use also appear to exist. Ciprofloxacin resistant *Campylobacter* have been found in Canadian pigs and on ABF farms in the United States (49,149,151-153). The ABF farms reported no fluoroquinolone exposure. No fluoroquinolones are licensed for use in Canada for pigs. It is possible that pigs in Canada could be exposed to fluoroquinolones if enrofloxacin (Baytril 100®), which is licensed for therapeutic use against respiratory disease in beef cattle, were used off-label or if fluoroquinolones were imported and used as active pharmaceutical ingredients (49). An on-farm study in Switzerland considered herd health variables as risk factors for AMR in *Campylobacter* from finisher pigs (154). Data quality prevented considering antimicrobial exposure, so herd health variables acted as a proxy. Every outcome (resistance to ciprofloxacin, erythromycin, streptomycin, tetracycline, and resistance to three or more drugs) was associated with health problems such as lameness, coughing and tail chews. The odds of ciprofloxacin resistance were three times higher in herds with tail-biting, even though fluoroquinolones are not a recommended treatment for this problem. However, the odds of ciprofloxacin resistance decreased with lameness in 100-kg pigs (Odds Ratio (OR) = 0.3) and coronary band lesions in 30-kg pigs (OR = 0.3).

Ciprofloxacin resistance was the only outcome that occurred less frequently in herds with health problems. These counter-intuitive risk factors may be the result of type I error; however, it is concerning that no biologically plausible risk factors were identified. These findings, along with ciprofloxacin resistant *Campylobacter* in fluoroquinolone naïve pigs in North America, raise questions about the source of fluoroquinolone resistance (148,149,152-154). Considering the public health importance of fluoroquinolone resistant *Campylobacter*, identifying risk factors for this resistance should be a priority for on-farm AMR studies.

Pork is a potential source of *C. coli* as the majority of healthy market-ready hogs are carriers (142,143). Although the role of pork in human infection is unknown, frequent resistance to macrolides and observed resistance to ciprofloxacin is extremely concerning (39,149,151). More on-farm studies are needed to identify risk factors for antimicrobial resistance in this potentially zoonotic pathogen.

2.7 Conclusions and Rationale for this Study

This review described the public health significance of antimicrobial resistant *Salmonella*, *E. coli* and *Campylobacter* in pigs, depicted the current situation on swine farms in North America, and discussed known risk factors for resistance. Developing on-farm control strategies for AMR requires more knowledge of how resistance varies regionally within Canada and between swine farms within a region. These findings, along with continued evaluation of on-farm risk factors, may identify production practices that either create unacceptable risks or could be changed easily and economically.

Together, Alberta and Saskatchewan produce more than 20% of Canada's market hogs (45). Despite this, the data describing AMR in western Canadian pigs are scarce. Only *Salmonella* from grow-finish pigs in Alberta, and *E. coli* from 10 swine herds in British Columbia, have been described. Antimicrobial resistance in *Campylobacter* has not been investigated on-farm anywhere in Canada. Finally, there is a lack of semi-quantitative antimicrobial exposure data in Canada. These data are needed to improve risk factor analyses describing antimicrobial use and resistance thus furthering understanding of the selective pressures created by antimicrobials for resistance.

Based on these knowledge gaps, this thesis was designed to address these research objectives:

- To describe the incidence of antimicrobial exposure of pigs on farms in Alberta and Saskatchewan.
- To describe the prevalence of phenotypic antimicrobial resistance in *Salmonella*, *E. coli* and *Campylobacter* from healthy pigs on farms in Alberta and Saskatchewan.
- To describe the association between antimicrobial use and phenotypic antimicrobial resistance in *E. coli* from healthy grow-finish pigs.
- To describe the genetic basis of antimicrobial resistance in *E. coli* from healthy grow-finish pigs.

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CHAPTER 3
ANTIMICROBIAL USE THROUGH FEED, WATER AND INJECTION IN PIGS ON-FARMS IN ALBERTA AND SASKATCHEWAN, CANADA

3.1 Introduction

Antimicrobial resistance is an animal welfare and public health problem; resistant bacterial infections are associated with greater morbidity, mortality and expense than their susceptible counterparts (1-4). Agricultural antimicrobial use (AMU) provides many benefits to livestock and producers including disease treatment, prevention and growth promotion (5). However, it also increases the prevalence of resistant bacteria, some of which are zoonotic (6-9). Despite this, descriptions of the quantities and reasons for antimicrobial use in Canadian livestock are scarce. This knowledge gap impedes our understanding of the link between antimicrobial use and antimicrobial resistance in food animals.

Antimicrobial use in North American pigs has been investigated through targeted research projects in Ontario and Alberta and national surveys in the United States (10-15). This project used on-farm records to describe the antimicrobial exposure incidence through feed, water and injection of suckling, nursery, grow-finish pigs and sows over 12 months.

3.2 Materials and Methods

3.2.1 Herd selection and data collection

Twenty farms were enrolled in the study by eight swine veterinarians in Saskatchewan (thirteen farms) and Alberta (seven farms). Each veterinarian enrolled two to four farms that met the study inclusion criteria of a minimum of 100 sows and enrollment in the Canadian Quality Assurance® (CQA®) Program (16). Each herd was visited once between May and September of 2004. A study veterinarian collected data from the herd owner or manager on antimicrobial use in the previous 12 months. Data were obtained from CQA® forms and by the completion of supplemental questionnaires that further described the antimicrobial use, herd inventory and animal flow through production (Appendix A). The data were organized using a relational database (Microsoft Access, Microsoft Corporation, Redmond, WA, USA) and descriptive statistics were calculated with Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA).

3.2.2 Feed and water (group) exposure

The following data were collected on antimicrobial exposures through feed or water in the previous 12 months: the production phase exposed, antimicrobial used, number of pigs exposed, duration of exposure, concentration of drug administered and reason for drug use. The group antimicrobial exposure incidence (AEI_G) per 1000 pig-days was determined for each use (Equation 1). Each day antimicrobials were offered was an exposure event. Every herd had open populations with animals entering and leaving the herd during previous 12 months. The pigs-at-risk was the average number of pigs moved into and out of each phase over this time. This assumed mortality and culls occurred, on average, halfway through each phase. The time-at-risk was the average

number of days spent in each phase. Time-weighted averages accounted for groups of pigs within a phase with different durations at risk. This occurred in herds where animals were sold as breeding stock or where batches of nursery pigs were sold at a younger age than the typical transfer age to grow-finish. Combination drug products were considered a single exposure regardless of the number of antimicrobials they contained with one exception. These exposures were stratified into the constituent drugs when describing exposure to individual antimicrobials.

Equation 1. Formula for Group Antimicrobial Exposure Incidence (AEI_G)

$AEI_G = [(Pigs_E * Days_E) / (Pigs_R * Days_R)] * 1000$

E = exposed

R = at risk

3.2.3 Parenteral (individual) exposure

Data on parenteral antimicrobial use in the previous 12 months were obtained through one of two methods. When existing records were unvavailable, a survey was administered by the study veterinarian (A.6). This survey collected data on the typical frequency of antimicrobial exposures, the most commonly used antimicrobials, typical dose, duration and reason for use. In herds with parenteral exposure records, data were entered into the database at the level of detail maintained in each herd. These data sources were used with herd inventory to calculate the parenteral antimicrobial exposure incidence (AEI_P) (Equation 2). All pig exposures to a drug within five days were considered a single exposure because the existing records in many herds only indicated if a pig had been exposed, not the number of times. This is was the only difference between

individual and group antimicrobial exposure incidences. In some herds, data were missing due to records having been lost or not maintained for portions of the previous twelve months. In these cases, the parenteral antimicrobial exposure incidence (AEI_P) was adjusted by multiplying the denominator by the percent of records available. Data were assumed to be missing at random.

Equation 2. Formula for Parenteral Antimicrobial Exposure Incidence (AEI_P)

$AEI_P = Pigs_E / [Pigs_R * Days_R * \text{percent records available}]$

3.2.4 Exposure by all routes

Total antimicrobial exposure for each phase, of each herd, was the sum of the feed, water and parenteral exposure incidences. On any day, a pig could be exposed by every route resulting in a maximum of three exposures per pig-day. Antimicrobial exposure by any route reflected the exposure by feed, water or injection and was the sum of these incidences constrained to a maximum of one.

3.2.5 Statistical analysis and data comparisons

Statistical models were adjusted for clustering in herds through generalized estimating equations (PROC GENMOD, SAS version 9.1 SAS Institute Inc., Cary, North Carolina). All models had a logit-link function, binomial distribution, exchangeable correlation structure, and were adjusted for the production phase exposed. Associations were reported as statistically significant if $P < 0.05$. The association between each outcome and variable of interest was reported as an odds ratio (OR = expβ) with 95% CI (17).

Binomial response models estimated the probability of exposure to an antimicrobial by any route, by feed, by water, or by injection for pigs in each production phase. The outcome was the number of exposure events in the numerator and the pig-days-at-risk in the denominator. Model convergence was a problem when estimating exposure by water. Therefore, this model was restricted to exposures of nursery pigs, grow-finish pigs and sows because no water exposure occurred in suckling piglets. The probability of exposure was estimated by the effect estimate (β) and 95% confidence intervals in the formula $1/[1 + \exp(-\beta)]$ (17). Significant pair-wise comparisons between phases, within routes, were noted.

The difference between the two methods used to collect parenteral AMU data was investigated by considering the data source, 'survey' versus 'existing records', as a predictor of AEI_P. Similarly, the possibility that the study inclusion criteria were associated with antimicrobial exposure was investigated. Two separate analyses examined the association of exposure by any route and with herd size and veterinary practitioner.

3.3 Results

Antimicrobial exposure was most frequent in nursery pigs. Exposure of grow-finish and suckling pigs was less than half that of nursery pigs. Antimicrobial exposure in sows was comparatively low (Table 3.1). Feed was the predominant route of administration for all production phases with 88% of the exposure incidence through

feed, 9% through water and 3% through injection. Grow-finish pigs in two herds were not exposed to any antimicrobials. In every other herd, pigs in all production phases had some antimicrobial exposure.

In every phase, the probability of exposure through feed was almost identical to exposure by all routes (Table 3.1). The probability of exposures by feed, and by all routes, was significantly different between every production phase except suckling piglets and grow-finish pigs. The probability of exposure through water was negligible for all phases except nursery pigs. This was reflected by a significantly higher probability of exposure in nursery pigs compared to the other production phases. Finally, the probability of parenteral antimicrobial exposure was significantly higher in suckling piglets than in other production phases.

3.3.1 Group exposure

Existing records were available in every herd describing the herd inventory and animal movement through each production phase. Additionally, in every herd CQA® records were available describing in-feed antimicrobial use. The herd representative was able to provide supplemental data beyond these records describing the administration of antimicrobials through feed and water. One herd representative reported no antimicrobial use through feed in any production phase. Of the other nineteen, all added antimicrobials to nursery diets, fifteen to grow-finish diets, ten to suckling piglet diets (commonly referred to as creep diets) and eight to sow diets (Table 3.1). Reflecting the pattern seen with exposure by all routes, the median exposure of nursery pigs was twice any other

production phase. Chlortetracycline, lincomycin, tiamulin and tylosin were the predominant drugs administered through feed (Table 3.2).

Producers reported more than 90% of the antimicrobials added to sow diets were to treat disease compared to less than 20% in the other pig phases (Table 3.3). The opposite occurred with AMU for disease prevention. Producers reported that roughly 80% of creep and nursery diet exposure was to prevent disease compared to less than 10% of the incidence in sows. The only production phase with substantial growth promotion AMU reported was grow-finish (Table 3.3).

Antimicrobials were administered by water in ten herds; six producers reported use in nursery pigs only, two in nursery and grow-finish pigs, one in grow-finish pigs only and one in sows. In herds with water antimicrobial use, the median nursery incidence was many times higher in than the other phases (Table 3.1). In nursery pigs, the predominant reason for water exposure was to prevent disease. The reported reason for all use in grow-finish pigs and sows was to treat disease (Table 3.3). In nursery and grow-finish pigs, the most commonly used antimicrobial was penicillin G (Table 3.4).

3.3.2 Parenteral exposure

The availability of parenteral exposure records differed markedly between herds. One herd had no existing records and one provided records for all production phases. Overall, on-farm records were supplied for parenteral exposure of suckling pigs in 1 herd, nursery pigs in 4 herds, grow-finish pigs in 19 herds, and sows in 12 herds. For all production phases, 12 months of data were collected from most herds. The minimum

collected was eight months. The data maintained differed between herds; all recorded the antimicrobial used, production phase exposed, and number of pigs exposed, while the dose and duration of exposure was variable. With one exception, producers unable to supply parenteral exposure records completed a survey describing their AMU. Data from one herd were insufficient to calculate the AEI_P in the nursery.

Every producer administered parenteral antimicrobials to suckling piglets and sows, one reported no use in nursery pigs, and five reported no use in grow-finish pigs. Suckling piglets were routinely injected in nine herds; all piglets were injected once in six herds, and twice in three herds. All sows were routinely injected with an antimicrobial after farrowing in one herd. For all phases, the parenteral exposure incidence was very low relative to exposure by feed or water (Table 3.1).

Although suckling piglets received the most parenteral antimicrobials, records were least available for this phase. Therefore, the most common products used in suckling piglets were described from survey data. Fourteen producers listed penicillin G as one of the two most common drugs used in their suckling piglets, nine listed trimethoprim-sulfadoxine and six listed ceftiofur. Other antimicrobials mentioned less frequently were oxytetracycline, spectinomycin and gentamicin. In the other phases, the description of parenteral products used was based on existing records becauset this allowed comparison based on the exposure incidence rather than the relative ranking by producers. Penicillin G was used in every herd and trimethoprim-sulfadoxine was used in

most (Table 3.5). Some antimicrobials were used in only a few herds, but were used extensively in those herds.

Study herds had a median of 456 sow (Inter-quartile range, 274 to 1042). Exposure by any route was not associated with the herd size ($P = 0.3$) or the veterinary practitioner ($P = 0.1$). The AEI_P was higher in herds providing data by survey compared to existing records (OR, 2.9; 95% CI, 1.5 to 5.6; $P = 0.002$).

3.4 Discussion

This study used on-farm records to describe the antimicrobial use in 20 swine herds in western Canada. Pigs received antimicrobials primarily by feed and secondarily by water. As data describing feed and water exposures were consistently available on every study farm, we determined that existing farm records are a feasible way to describe group medication rates. A previous study described injectable exposure rates from purposively collected data (12). Demonstrating that sufficient on-farm data were available to calculate a rate-based description of AMU for group exposures is an important advancement for describing antimicrobial use in Canadian pigs.

Antimicrobial use differed significantly between the production phases. Biological differences likely account for the high parenteral exposure of suckling piglets compared to older animals. Feed and water dosage regimens are inappropriate for treating disease in suckling piglets because consumption is low and variable (18). Both biological and production differences could explain the higher exposures in nursery and grow-finish pigs compared to breeding stock. Growing animals are more susceptible to many

respiratory and gastrointestinal infections due to waning maternal immunity, mixing and other stressors. Additionally, growing animals may be exposed to antimicrobials to improve growth (18,19). To address these differences, AMU guidelines must be production phase specific. When evaluating the effect of guidelines on AMU, data should be stratified by phase because herd-level estimates could mask substantial phase-level changes.

The high probability of nursery pig exposure raises two food safety concerns. First, pigs marketed substantially below the typical live-weight of 110 kg (commonly known as barbeque hogs) exceed maximum drug residue limits more often than market age animals or sows (20). Second, antimicrobial exposure in nursery pigs is associated with increased antimicrobial resistance in market age animals (8,21-23). Producer education on responsible drug use appears to have decreased residue violations (20). This suggests education has the potential to change AMU practices associated with antimicrobial resistance.

Not only did AMU vary between production phases, it also varied substantially between herds, within phases. Most notably, the grow-finish feed AEI ranged from 0 to 1000 exposures per 1000 pig-days. This is partially explained by growth-promotional use, which could be a target for judicious use campaigns since benefits decrease as pigs age (19). Variation between herds could also be due to different disease pressures, facility designs and production practices. Investigating if these factors are associated with AMU might identify practical interventions to decrease pig exposures.

Tylosin use in feed was notable considering macrolides are classified as being of 'high' or 'critical' importance to human health (24-26). Given the variable exposures between herds, targeted judicious-use campaigns might alter the use of this drug. However, before efforts to change drug use practices are undertaken, veterinarians, producers and policy-makers need to know if effective alternatives are available. This ensures the continued health and welfare of the livestock that may be affected by a drug ban or changes in label use.

The AEI_G illustrated the relatively minor contribution of water antimicrobials to mass medication exposure; a fact that was not elucidated by previous qualitative descriptions (13,14). This project did not investigate the appropriateness of antimicrobial use. However, using penicillin G in water warrants comment because it is largely degraded in gastric acid and poorly absorbed with oral administration (27,28). Swine veterinarians have traditionally recommended penicillin G because alternative beta-lactam formulations, such as penicillin V, are not registered for use in food animals in Canada, and amoxicillin has just recently been registered for swine in Canada (29). This suggests that limited treatment choices can result in inappropriate AMU.

The AEI_G had two limitations. First, it was a semi-quantitative measure of AMR as it did not reflect the amount of antimicrobials consumed. Study farms provided data on the duration of antimicrobial exposures but not the pig weights or feed / water consumption estimates. This precluded calculating the amount of antimicrobials

consumed in study herds. Others have applied standard animal weights and feed budgets to similar data to approximate consumption (30). In contrast, AMU was presented as an incidence for two reason: to demonstrated the available on-farm data and to avoid imprecise exposure estimates. The precision of the exposure estimates was important because the antimicrobial exposure data were used in risk factor analyses that assume no error in the predictor variables (Chapter 7 and 8) (17). The second limitation of the AEI_G was it described the intention to expose pigs to antimicrobials and assumed every animal consumed the offered feed or water. This means creep feed estimates overstated exposure because consumption in suckling piglets is low and variable, and feed wastage is not accounted for (18). Future studies should consider validating exposure data collected from existing records by comparing reported values to feed tags, invoices or disappearance. This study was unable to validate drug-use estimates as the data were collected retrospectively.

Describing parenteral exposure was more challenging than feed or water exposure. Parenteral AMU records for nursery and suckling piglets were not available in most herds because, at the time of data collection, the CQA® program required producers to maintain records only for animals over 22 kg (16). Sow exposure data were not available in some herds because records were maintained on sow cards that traveled with the sow and were often not retained between parities. In addition to a lack of records, two concerns arose with estimating AEI_P from existing records. First, the records supplied by most herds only indicated that pigs were exposed, not the number of exposures in the treatment regimen. This made it impossible to describe multiple exposures to a product

within five days. Secondly, AEI_P was significantly different depending on the data source: the odds of exposure were three times higher for pigs in herds reporting AEI_P by survey than by existing records. Although the surveys could have overestimated use, others have found treatment records underestimated injectable use by 35% compared to inventory disappearance (12). It is plausible that the existing records underestimated the AEI_P because these were often kept in the rooms with the pigs and would be subject to loss and damage. Additionally, in times of staff shortages or disease outbreaks it is plausible that treatments may not be recorded.

The herd representatives that complete parenteral antimicrobial use surveys described the typical exposure rates, reasons for antimicrobial use and doses. However, antimicrobial use may be inconsistent across seasons or groups of pigs. The producer's estimate of drug use could be biased by the current rate of use in the barn: resulting in an over or under estimation of the true exposure incidence. As the study was retrospective, we were unable to assess the extent of this potential bias. The second limitation of the parenteral exposure survey was the failure to describe the proportion of treatment incidence attributable to different antimicrobials. Producers listed the two most commonly used antimicrobials in the production phase as well as the first and second choice treatments for common disease problems. However, data were insufficient to estimate the exposure incidence by antimicrobial. This was a serious limitation: it limited the number of herds in the description of AEI_P by product and precluded considering AEI_P in risk factor analyses for AMR (Chapter 7 and 8).

Considering the small contribution of parenteral exposure to total use, it is unlikely that bias from existing records substantially affected total exposure estimates. However, in light of the problems identified with collecting parenteral exposure data, future studies should attempt to validate existing records or seek alternative methods for estimating parenteral antimicrobial exposure.

The exposure incidence to antimicrobials that were used in both parenteral and oral forms was dominated by the feed and water exposure; parenteral exposure could be considered negligible relative to oral exposure. However, quantifying the exposure to drugs used only as parenteral products, such as ceftiofur and trimethoprim-sulfadoxine, was valuable. The World Health Organization (WHO) considers third generation cephalosporins critically important for human health, thus researchers may be interested in evaluating the associations between ceftiofur use and AMR (25). The relatively low rates of exposure, and number of herds with no use, indicate case-control studies would be a feasible way to investigate such associations (17).

The herd selection, and the study inclusion criteria, could have affected AMU estimates. However, given that the associations between antimicrobial exposure and veterinarian and herd size were not significant, it is unlikely that the inclusion criteria substantially biased AMU estimates. This study was limited to herds with more than 100 sows to better represent market hog production in western Canada. Although 70% of Saskatchewan pig farms marketed less than 1000 pigs in 2004 (which roughly corresponds to herds of 50 sows or less) these farms marketed less than 3% of

Saskatchewan's pigs (31). The herds enrolled in this study were a convenience sample based on the presence of a veterinary – client – patient (VCP) relationship. This could be important considering, in the United States, producers with a VCP relationship were more likely to use feed grade antimicrobials than those without (32). All study herds were enrolled in the CQA® program (16). While this may have affected AMU, the herds were reflective of western Canada at the time of the study: 98% of market hogs produced in Alberta and 99.8% in Saskatchewan came from herds enrolled in the CQA® program **(**Personal communication: Harvey Wagner, Sask Pork; Sarah Turner, Alberta Pork).

The semi-quantitative description of AMU made it difficult to compare these data to previous reports. However, the proportion of herds with antimicrobials administered by each route, and the most commonly used products, were similar to an Alberta based survey of 90 swine herds conducted in 2000 (14). Combining the detailed, semi-quantitative data from this study with the robust, but qualitative, data from the previous larger study provides a detailed account of antimicrobial use in this important swine-producing region of Canada (14). Future research should investigate if descriptions of exposure incidence, common dosage regimens, and feed budgets can be applied to qualitative data to estimate the volume of antimicrobials used in Canadian pigs. If validated, authorities could efficiently respond to international recommendations that countries monitor and report antimicrobial use in livestock (33,34).

This description of feed and water antimicrobial exposure incidence is unique in Canadian pigs. This project illustrated the variability in AMU between phases and herds.

Identifying the reasons for these variations might focus future interventions to decrease AMU. Most importantly, this study demonstrated that on-farm records were sufficient to describe pig antimicrobial exposure through feed and water.

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Table 3.1 Probability and 95% confidence intervals of antimicrobial exposure by a given route per pig-day (all, n = 80 (20 herds with four production phases per herd); feed, n = 80; injection, n = 79; water, n = 60 (20 herds with three production phases per herd)) and distribution of the antimicrobial exposure incidences per 1,000 pig-days of each production phase in herds with an incidence greater than zero (N = 20 herds).

Phase	Administration Route*	Probability of exposure	95% confidence interval	AEI in herds reporting use		
				Herds	Median	IQR**
Suckling	All routes [b,d]	0.21	0.08 - 0.46	20	95	34 - 403
	Feed [b,d]	0.17	0.05 - 0.44	10	333	276 - 492
	Water	-	-	0	-	-
	Parenteral [b,c,d]	0.04	0.02 - 0.07	20	29	11 - 57
Nursery	All routes [a,c,d]	0.79	0.56 - 0.92	20	677	327 - 1019
	Feed [a,c,d]	0.78	0.55 - 0.92	19	667	394 - 1000
	Water [c,d]	0.06	0.02 - 0.19	8	200	125 - 514
	Parenteral [a]	0.001	.0004 - .002	18	1	0 - 1
Grow-finish	All routes [b,d]	0.31	0.16 - 0.51	18	455	108 - 927
	Feed [b,d]	0.31	0.16 - 0.51	15	714	160 - 977
	Water [b]	0	0 - 0.001	3	5	3 - 8
	Parenteral [a,d]	0.0003	.0001 - .0009	15	0	0 - 0
Sow	All routes [a,b,c]	0.03	0.01 - 0.09	20	1	0 - 21
	Feed [a,b,c]	0.03	0.01 - 0.09	8	49	15 - 197
	Water [b]	0	0 - 0.004	1	7	-
	Parenteral [a,c]	0.001	.0004 - 0.002	20	1	0 - 1

* All routes: the probability describes exposure by any route (maximum probability of one) while median and IQR describes the total antimicrobial exposure (maximum of three per pig-day)
** IQR: Inter-quartile Range
a: different from suckling piglets ($P < .05$)
b: different from nursery pigs ($P < .05$)
c: different from grow-finish pigs ($P < .05$)
d: different from sows ($P < .05$)

Table 3.2 Antimicrobial exposure incidence per 1,000 pig days through feed, by product, in herds with an exposure incidence greater than zero (N = 20).

		Feed antimicrobial exposure incidence		
Phase	Antimicrobial	Herds	Median	IQR
Suckling	Chlortetracycline	5	333	232 - 455
	Tiamulin	4	184	29 - 465
	Lincomycin	3	700	333 - 778
	Spectinomycin	3	700	333 - 778
	Penicillin G	3	286	190 - 333
	Sulfonamides*	3	286	190 - 333
	Tylosin	1	286	-
Nursery	Chlortetracycline	13	226	198 - 614
	Lincomycin	10	433	248 - 826
	Tiamulin	9	264	190 - 475
	Spectinomycin	6	319	137 - 525
	Tylosin	5	316	257 - 750
	Penicillin G	4	175	126 - 221
	Sulfonamides*	4	175	126 - 221
	Tilmicosin	1	200	-
	Oxytetracycline	1	173	-
	Neomycin	1	173	-
Grow-finish	Tylosin	11	500	111 - 953
	Lincomycin	5	697	217 - 1000
	Chlortetracycline	1	720	-
	Tilmicosin	1	52	-
	Penicillin G	1	24	-
	Sulfonamides*	1	24	-
Sow	Oxytetracycline	3	37	16 - 233
	Chlortetracycline	2	99	-
	Lincomycin	2	18	-
	Tylosin	1	300	-
	Penicillin G	1	64	

* the specific sulfonamide used was not available in all herds

Table 3.3 Percent of group antimicrobial exposure incidence (AEI_G) according to producer-declared reason for antimicrobial use.

		Percent group antimicrobial exposure incidence		
Route	Phase	Disease Treatment	Disease Prevention	Growth Promotion
Feed	Suckling	18.5	81.5	0.0
	Nursery	12.9	79.9	7.2
	Grow-finish	10.8	47.1	42.1
	Sow	93.0	7.0	0.0
Water	Nursery	13.6	86.4	-
	Grow-finish	100.0	0.0	-
	Sow	100.0	0.0	-

Table 3.4 Antimicrobial exposure incidence per 1,000 pig-days through water, by product, in herds with a treatment incidence greater than zero (N = 20).

		Water antimicrobial exposure incidence		
Phase	Antimicrobial	Herds	Median	1QR
Nursery	Penicillin G	4	252	194 - 823
	Neomycin	3	71	12 - 600
	Sulfonamide*	2	108	-
	Tetracycline	1	600	-
	Amoxicillin	1	194	-
Grow-finish	Penicillin G	2	6	-
	Tetracycline	1	5	-
Sow	Tetracycline	1	7	-

* the sulfonamide derivative was not available in all herds

Table 3.5 Parenteral antimicrobial exposure incidence per 1,000 pig days, by product, in herds with an exposure incidence greater than zero and providing data by existing records (Nursery N = 4, Grow-finish N = 19, Sow N = 12).

		Parenteral antimicrobial exposure incidence		
Phase	Antimicrobial	Herds	Median	IQR
Nursery	Penicillin G [a]	4	0.14	0.02 - 0.56
	Trimethoprim-sulfadoxine	4	0.04	0.01 - 0.45
	Oxytetracycline	4	0.04	0.01 - 0.18
	Ceftiofur	2	0.16	-
	Lincomycin	2	0.04	-
Grow-finish	Penicillin G	14	0.08	0.01 - 0.41
	Trimethoprim-sulfadoxine	8	0.03	0.01 - 0.18
	Ceftiofur	7	0.01	0.002 - 0.03
	Oxytetracycline	5	0.05	0.00 - 0.10
	Lincomycin	4	0.14	0.01 - 0.45
	Tylosin	3	0.001	0.000 - 0.004
	Tiamulin	1	0.22	-
	Ampicillin	1	0.1	-
Sow	Penicillin G	12	0.16	0.06 - 0.46
	Trimethoprim-sulfadoxine	9	0.11	0.02 - 0.23
	Oxytetracycline	8	0.2	0.01 - 0.61
	Tylosin	4	0.05	0.01 - 0.11
	Ceftiofur	3	0.004	0.002 - 0.01
	Lincomycin	2	0.23	0.01 - 0.45
	Ampicillin	1	0.03	-

a: procaine and benzathine penicillin G use could not be distinguished

CHAPTER 4
ANTIMICROBIAL RESISTANCE OF FECAL *SALMONELLA* SPP. ISOLATED FROM ALL PHASES OF PIG PRODUCTION IN 20 HERDS IN ALBERTA AND SASKATCHEWAN, CANADA

4.1 Introduction

Salmonella spp. are second only to *Campylobacter* spp. as the leading cause of foodborne disease in Canada. Approximately 6,000 to 9,000 cases of human salmonellosis are reported annually, and for each reported illness 13 to 37 cases remain unreported (1-3). Antimicrobial resistant *Salmonella* can cause even greater morbidity than their susceptible counterparts due to treatment failure, increased infection severity, and increased rates of disease in people taking antimicrobials for other reasons (4-7). Although pork is not a major cause of salmonellosis in North America, it has been responsible for disease outbreaks of multiresistant *Salmonella* in humans elsewhere (8-10).

Most *Salmonella* infections are acquired from contaminated food. Therefore, studying antimicrobial resistance (AMR) in live, close-to-market pigs indirectly estimates the potential for carcass contamination at slaughter and the risk to consumers from resistant organisms in pork (11,12). In Canada, *Salmonella* AMR data are available from pigs at slaughter (13-17). However, these data may differ from those acquired on-farm because AMR patterns and serovars can change after transport and lairage (18). Extending AMR investigations to other pig categories, such as sows and nursery pigs, might improve our understanding of the occurrence and spread of AMR within pig production systems. This could lead to identifying control measures. Currently, on-farm *Salmonella* AMR data from healthy pigs in Canada are limited to a longitudinal study of

finishing pigs from Alberta (19). The main goal of this study was to investigate and describe the AMR profiles of *Salmonella* isolates from apparently healthy nursery pigs, grow-finish pigs and sows in 20 herds in Alberta and Saskatchewan, Canada.

4.2 Materials and methods

4.2.1 Herd and sample selection

A convenience sample of 20 farms was allocated to eight swine veterinarians in Saskatchewan (13 farms) and Alberta (7 farms). Farms were selected by veterinarians based on a minimum size of 100 sows and enrollment with the Canadian Quality Assurance® Program (20). The number of farms per veterinarian ranged from two to four. Half of the veterinarians were asked to identify the presumed *Salmonella* status of the herds. Ten herds were enrolled with knowledge of the presumed *Salmonella* status; seven *Salmonella*-positive and three *Salmonella*-negative (based on clinical disease or test results in the previous 12 months) were selected to address the needs of a separate research project. The principle investigator and laboratory personnel were blind to the presumed herd status. The remaining 10 herds, with an unknown *Salmonella* status, were selected from as many veterinarians as possible in order to minimize clustering by geography and management practices.

Each herd was visited once between May and September 2004. Fresh fecal samples were collected from pens, which were selected using a random numbers table. Pooled pen samples (PPS) consisted of freshly voided feces from five pigs for a pooled sample weight of approximately 75 g. In the 10 herds of unknown *Salmonella* status, 25 PPS were collected from grow-finish pigs. In the 10 presumed-known *Salmonella* status herds, samples were collected from each phase of pig production: 20 PPS from sows, 30 PPS from nursery pigs, and 30 PPS

from grow-finish pigs. Sow samples were collected from both the farrowing and gestation stages. Although each pooled sample contained feces from five sows, animals were often housed in individual crates. In addition, 30 individual-animal samples were collected from grow-finish pigs and 10 from sows (Figure 4.1). The intensive sampling strategy was implemented on presumed known-*Salmonella* status farms because investigating differences in AMR between production phases was a secondary objective to the primary study objective of describing *Salmonella* AMR on swine farms in Alberta and Saskatchewan.

4.2.2 Laboratory methods

***Salmonella* isolation.** All samples were shipped on ice to one of three laboratories within 24 h of collection. Samples from ten herds were cultured for *Salmonella* by Agri-Food Laboratories Branch (AFLB), Food Safety Division of Alberta Agriculture, Food and Rural Development, Edmonton, AB; samples from four herds by Prairie Diagnostic Services (PDS), University of Saskatchewan, Saskatoon, SK; and samples from six herds by Laboratory Services Division, University of Guelph, Guelph, ON.

AFLB Food Safety Division of Alberta Agriculture – Edmonton, AB

Salmonella Typhimurium ATCC 14028 was used as the quality control organism. Unless otherwise specified, the incubation temperature was 35° C. A 10 g fecal aliquot per sample was pre-enriched in 90 ml of Buffered Peptone Water (BPW) for 20 to 24 h. From the BPW, 0.1 ml was transferred into 10 ml Rappaport Vassiliadis (RV) enrichment broth and incubated at 42° C for 24 h. Concurrently, 1.0 ml was transferred into 10 ml of Tetrathionate (TT) enrichment broth containing 0.2 ml of iodine solution and incubated for 24 h. Aliquots from the RV and TT broths (0.15 ml each) were pooled and screened by real-time polymerase chain reaction (RT-PCR) for

the presence of *Salmonella* (21). Following incubation, 10 µl of RV broth and 10 µl of TT broth were transferred to Xylose-Lysine-Tergitol 4 Agar (XLT4) and Rambach Agar (RAM) plates and incubated for 24 to 48 h. The TT broth (0.1 ml) was transferred to three sites on Modified Semi-Solid Rappaport Vassiliadis (MSRV) (Difco) media and incubated at 42° C for up to 72 h. Each MSRV plate with a halo was subcultured onto XLT4 and RAM plates. Samples testing positive by RT-PCR, but culture negative, were tested with ImmunoMagnetic Separation (IMS) technology (Dynabeads™ anti-*Salmonella,* Dynal® Biotech, ASA, Oslo, Norway). Beads were enriched in TT broth and processed as described above. In general, one suspect *Salmonella* colony per sample was selected for further characterization unless morphologically different colonies were identified, in which case both were harvested. Isolates were initially screened with triple sugar iron agar (TSI), lysine iron agar (LIA), urea agar slants and purity checked using one-quarter MacConkey agar and blood agar plates respectively. Isolates were further screened with *Salmonella* Poly 0/01 agglutination (Denka Seiken Co. Ltd. Japan) and atypical colonies were tested with Vitek GNI or API-20E (bioMérieux Canada, Inc.). Presumptive *Salmonella* isolates were frozen at -70° C in defibrinated sheep's blood and sent to the Office International des Épizooties (OIÉ) Reference Laboratory for Salmonellosis, Guelph, ON for confirmation by serotyping.

Prairie Diagnostic Services – University of Saskatchewan – Saskatoon, SK

The protocol developed by AFLB was utilized by PDS with minor modifications; RT-PCR and IMS were not used. Screening differed in that suspect colonies were incubated for 2 to 4 h in trypticase soy (TS) broth and subsequently tested with a citrate slant rather than LIA. Isolates were sent in TS broth to the Saskatchewan Health Provincial Laboratory for serotyping.

Laboratory Services Division – University of Guelph – Guelph, ON

Health Canada's standard *Salmonella* isolation protocol was used with the IMS technology (Dynabeads™ anti-*Salmonella,* Dynal® Biotech, ASA, Oslo, Norway) (22). Suspect colonies were tested with TSI, LIA and urea slants to confirm *Salmonella* status, frozen at -80° C in glycerol, and sent to the OIÉ Reference Laboratory for Salmonellosis, Guelph, ON for serotyping.

Serotyping and phagetyping

Office International des Épizooties (OIÉ) Reference Laboratory for Salmonellosis, Guelph, ON

The O antigens of the *Salmonella* isolates were determined by slide agglutination (23). The H antigens were identified using a microtechnique that employs microtitre plates (24). The antigenic formulae of Le Minor and Popoff were used to name the serovars (25). The standard phagetyping technique described by Anderson and Williams was followed (26). The phagetyping scheme and phages for *Salmonella* Typhimurium, developed by Callow, and further extended by Anderson, and Anderson and colleagues, were obtained from the International Centre for Enteric Phage Typing (ICEPT), Central Public Health Laboratory, Colindale, United Kingdom via the National Microbiology Laboratory (NML), Public Health Agency of Canada, Winnipeg, Manitoba (27-30). The *Salmonella* Heidelburg phagetyping scheme and phages were supplied by the NML (31). Isolates that reacted with the phages but did not conform to any recognized phage type were considered atypical. Strains that did not react with any of the typing phages were considered untypable.

Saskatchewan Health Provincial Laboratory – Regina, SK

The O antigens of the *Salmonella* isolates were determined by slide agglutination and the H antigens were identified by a broth culture method (32). The antigenic formulae of Le Minor and Popoff were used to name the serovars (25). Samples identified as *S*. Typhimurium (including var. Copenhagen), *S*. Heidelberg or *S*. Enteriditis were sent to the OIÉ Reference Laboratory for Salmonellosis, Guelph, ON for phagetyping as described above.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was conducted by AFLB and PDS using a broth microdilution technique following Clinical and Laboratory Standards Institute (CLSI) guidelines (33,34). National Antimicrobial Resistance Monitoring System (NARMS) minimum inhibitory concentration (MIC) susceptibility panels CMV7CNCD (Sensititre™, TREK Diagnostic Systems, Westlake, Ohio), were used to test isolates for susceptibility to 16 antimicrobials across a standard range of dilutions (Figure 4.2). Each isolate was grown up on a non-selective media. A 0.5 McFarland standard was made in 5 ml of demineralized water, of which 10 µl was transferred into 11 ml of cation-adjusted Mueller-Hinton broth with TES buffer. A 50 µl aliquot was inoculated into each of the 96 wells on the panel. Inoculated plates were incubated and read by the Sensititre ARIS® (Automated Reading and Incubation System) (TREK Diagnostic Systems, Inc., Westlake, Ohio). Readings were transferred to Sensititre® Automated Microbiology Systems (SAMS) (TREK Diagnostic Systems, Inc.) computer software and interpreted according to CLSI breakpoints for animals or humans (33,34) (Figure 4.2). The MIC breakpoints for streptomycin, which does not have CLSI guidelines, were taken from the National Antimicrobial Resistance Monitoring System

(NARMS) 2003 *Salmonella* report (15). Quality control organisms used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeroginosa* ATCC 27853.

4.2.3 Data comparisons and statistical analysis

Culture, serovar and MIC data were maintained in a relational database (Microsoft Access, Microsoft Corporation, Redmond, Washington, USA). Intermediate MIC values were categorized as susceptible for all analyses (33,34). Isolates susceptible to all drugs in the panel were designated 'pansusceptible'. This definition does not imply isolates are susceptible to drugs not on the panel. Isolates resistant to two or more drugs were designated 'multiresistant' (14,19,35).

Descriptive analyses were conducted using commercially available software (Microsoft Excel, Microsoft Corporation, Redmond, Washington, USA). Statistical analyses were performed with generalized estimating equations (GEE) to account for clustering of resistance within herds (PROC GENMOD, SAS for Windows version 9.1, SAS Institute Inc., Cary, North Carolina). All models had a binary outcome, logit-link function, and an exchangeable correlation structure.

Null binomial response models estimated the prevalence of pansusceptibility, multiresistance and resistance to each drug. From each model the intercept (β_0) and 95% confidence intervals (CI) were used to calculate population-average prevalence estimates using the formula $[1 + \exp(-\beta_0)]^{-1}$ (36). Univariate logistic regression examined the source of isolates

(production phase) as a predictor for the following outcomes: resistance to each drug, pansusceptibility and multiresistance.

Four additional sets of univariate analyses explored potential biases introduced by study design. The factors known prior to susceptibility testing, presumed known versus unknown herd *Salmonella* status, herd size, individual sample versus PPS, and laboratory performing isolation were each considered as predictors for drug resistances observed in more than 5% of the isolates. Associations between variables of interest and outcomes were reported as an odds ratio (OR = expβ) with 95% CI. All associations with $P < 0.05$ were reported as significant (36).

4.3 Results

Salmonella was identified in 14 herds with a range of 2 to 95 positive samples per herd. Overall, 32% of the samples were positive for *Salmonella* and 16 samples had two distinct isolates harvested, resulting in 468 isolates for susceptibility testing. Thirty serovars were identified with nine accounting for 89% of the isolates (Table 4.1). These nine serovars were each identified in at least two herds and all except serovar Banana were found in every production phase (Table 4.2).

Most of the *Salmonella* tested (293) were pansusceptible. Fifty-one isolates were resistant to one drug on the test panel, 27 were resistant to two, 24 were resistant to three, 61 were resistant to four, 10 were resistant to five, and two isolates were resistant to six of the sixteen drugs on the test panel. The highest prevalence of resistance was to tetracycline. Every tested isolate was susceptible to five of the drugs on the panel (Figure 4.2). All isolates also appeared susceptible to amikacin but the status of one isolate was indeterminable because the dilution

range did not cross the breakpoint and the MIC was greater than the dilution range. More than 85% of the isolates from six of the nine most common serovars were pansusceptible (Table 4.1). In contrast, only 8% of *S.* Mbandaka, 20% of *S.* Typhimurium var. Copenhagen, and 55% of *S.* Derby were pansusceptible (Table 4.1).

Salmonella was isolated from 32% of the nursery samples, 28% of the grow-finish samples and 47% of the samples from sows (Table 4.3). A higher percent of nursery, compared to grow-finish or sow, isolates demonstrated resistance. This pattern was evident across all drugs except trimethoprim-sulfamethoxazole, cefoxitin, and gentamicin (Table 4.4). The probability of observing resistance to four of the drugs was significantly higher in isolates obtained from nursery pigs than from grow-finish pigs. Likewise, the probability of an isolate demonstrating resistance to two of the drugs was higher if obtained from nursery pigs than from sows (Table 4.4).

Overall, 59% (95% CI, 42 to 75) of the *Salmonella* were pansusceptible. The majority of isolates from sows (74%; 95% CI, 53 to 88) were pansusceptible, as were approximately half of the isolates from grow-finish (56%; 95% CI, 36 to 73) and nursery pigs (48%; 95% CI, 26 to 71). The odds of an isolate being pansusceptible were significantly higher in sows compared to the other production phases (Table 4.4). Multiresistance was observed in 29% (95% CI, 16 to 48) of the isolates. The prevalence of multiresistance was higher in isolates from nursery pigs (48%; 95% CI, 26 to 71) than from grow-finish pigs (26%; 95% CI, 13 to 46) or sows (22%; 95% CI, 10 to 42). The difference between *Salmonella* from nursery pigs and sows was statistically significant (Table 4.5).

Twenty-nine resistance patterns were identified and nine occurred in more than five isolates (Table 4.6). Eight of these nine were found in more than one phase in a herd. Despite this, isolates with the same pattern, from the same phase, and same herd, were not always the same serovars. Unobserved resistance patterns were also notable; resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT), suggestive of a chromosomally located gene cluster (37), was not identified in any isolate. Also noteworthy, the 13 *S.* Typhimurium var. Copenhagen phage type 104 isolates were all pansusceptible.

Herd size was associated with resistance to streptomycin; the odds of resistance increased 1.02 times (95% CI, 1.01 to 1.03; $P = 0.003$) for every 1000 pigs marketed annually. No other resistance outcome was significantly associated with herd size ($P > 0.05$). Sample type was a significant predictor of two resistance outcomes. The odds of resistance to kanamycin were 1.2 times higher (95% CI, 1.1 to 1.3; $P < 0.0001$) in isolates from individual animals compared to PPS. In contrast, the odds of streptomycin resistance were decreased 0.47 times (95% CI, 0.26 to 0.86; $P = 0.02$) in samples collected from individual animals. Resistance to ampicillin, chloramphenicol, sulfamethoxazole and tetracycline were not significantly associated with sample type ($P > 0.1$). Finally, knowledge of the presumed herd *Salmonella* status and laboratory performing isolation were not significantly associated with resistance to streptomycin, sulfamethoxazole or tetracycline ($P > 0.2$). For resistance to ampicillin, chloramphenicol, and kanamycin, the significance of these variables as predictors of resistance could not be determined because all isolates from unknown-status herds were susceptible.

4.4 Discussion

Describing AMR in *Salmonella* from live pigs reflects the food safety risk for contaminated pork while providing insight into the epidemiology of resistance on-farms. Different rates of resistance in each production phase shows *Salmonella* from each pig-class may pose unique food safety risks and suggests that resistance is dynamic within barns. Hence, future on-farm studies should investigate risk factors for resistance in each production phase. Identifying variables associated with changes in resistance between phases might lead to interventions to mitigate AMR in *Salmonella*.

The prevalence of pansusceptible isolates was consistent with previous reports from Canada (40 to 53%), and higher than observed in the United States (6%) (13,16,19,38). Resistance to both tetracycline and sulfamethoxazole was also similar to previous Canadian studies (tetracycline, 38 to 49%; sulfamethoxazole, 21 to 36%) (13,14,16,19). These Canadian data contrast with numerous United States studies, which have found more than 80% of *Salmonella* were resistant to tetracycline (17,35,38). Such differences could be partially attributable to serovar; in 2004 CAHFSE described 59% of the tested *Salmonella* as *S.* Derby or *S.* Typhimurium (including Copenhagen) while the top two serovars identified by CIPARS (Derby and London) accounted for only 31% of the *Salmonella* isolates. Annual reports for both programs noted that AMR patterns were related to serovar. However, other factors in addition to serovar must be considered as the two most common serovars in this study matched those described by CAHFSE.

Antimicrobials included on the susceptibility test panels were selected based on their importance in human, rather than swine, medicine. Hence, resistance to numerous drugs were

examined which are not used in Canadian pigs including amikacin, cefoxitin, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, kanamycin and nalidixic acid (39). Despite the lack of pig exposures to these drugs, there was notable resistance to kanamycin and chloramphenicol. This study was not designed to explain the source of these resistances; co-selection, cross-resistance, and clonal resistance are all possible explanations. However, these findings emphasize the complexity of antimicrobial resistance in livestock. Finding resistance to drugs not used in pigs illustrates that the risk factors for resistance can be complex and multifactorial.

Fluoroquinolones are used to treat invasive salmonellosis in humans, while third-generation cephalosporins are indicated for *Salmonella* infections in children. There are few therapeutic alternatives to these antimicrobial classes (12,40). Because humans generally acquire resistant *Salmonella*, rather than the resistance developing during treatment, resistance to these drugs in isolates from food animals is of utmost importance (12). Like other North American reports, no resistance was noted to ceftriaxone, ciprofloxacin or nalidixic acid (15,16,19,38). Likewise, no resistance was found to ceftiofur, which contrasts with a report of frequent resistance on United States farms (21%) (38). Over 75% of the ceftiofur resistant *Salmonella* described by CAHFSE were serovar Derby (38). Thus, as Canadian swine abattoir surveillance has noted changing serovar distributions over time, this report of ceftiofur resistance in the United States suggests Canadian authorities and the swine industry should be vigilant for emerging third-generation cephalosporin resistance in *Salmonella* from pigs (38,41).

Antimicrobial resistance studies typically focus on close-to-market pigs to reflect consumer-risk from contaminated pork (17,19). However, people can also be exposed to resistant

bacteria from pigs through direct contact or contaminated environments (42,43). Additionally, resistant bacteria in other pig classes are also a potential food safety hazard. Young animals are slaughtered in provincially regulated abattoirs for local consumption (44). Culled breeding stock from Canada are predominantly slaughtered in the United States, where this class accounts for approximately five percent of slaughtered pigs (45,46). In addition to providing a food safety perspective, information on resistance within pig categories provides insight into AMR within farms. Nollet et al. also observed less resistance in *Salmonella* from sows compared to growing pigs, but did not corroborate our observation of increased resistance in nursery pigs (47). Others have reported relatively more resistance in coliforms from young pigs (48,49). These reports speculate that young animals carry more resistant organisms due to increased antimicrobial exposure and physiological differences (48,49). Identifying risk factors for resistance in nursery pigs is crucial; weaned pigs commonly receive antimicrobials, and in some instances are continuously exposed to drugs, which raises concerns about selection for resistance (50,51) (Chapter 3). Determining if antimicrobial use in nursery pigs is a risk factor for AMR in *Salmonella* should be a research priority: low-risk drug use practices could be incorporated into judicious use guidelines.

Elevated rates of AMR in nursery pigs may also be important in the spread of resistance between and within herds. Pigs are often mixed at transition to the grow-finish stage, either with other pens or with pigs from other farms. This creates an opportunity for *Salmonella,* and AMR genes, to spread to new animals. Despite this, there was a lower rate of resistance in *Salmonella* from grow-finish pigs. This suggests that resistant *Salmonella* populations may be unstable

within barns. Therefore, there are great potential benefits if studies can explain why *Salmonella* resistance declines as pigs progress to market.

Describing how resistance in *Salmonella* related to herd and phase was complicated by the relationship with serovar. The most common resistance pattern in this study (ampicillin-kanamycin-sulfamethoxazole-tetracycline) occurred only in *S*. Typhimurium var. Copenhagen, and only in two herds, although *S*. Typhimurium var. Copenhagen occurred in four other herds without this pattern. Describing the relatedness of these isolates might clarify if this resistance was due to common selective pressures or the spread of a resistant clone. Such information could prove important, as the control efforts to prevent the development of resistance could differ from those aimed to suppress a dominant clone. In numerous situations, *Salmonella* with the same resistance pattern and serovar were found in different production phases of the same herd. Thus, also describing the relatedness of isolates from different production phases could improve our understanding of how resistance spreads and persists within farms. Regardless of how these isolates were related, the apparent clustering of serovars and resistance patterns within herds helped to put the food safety risk from these *Salmonella* in perspective. For example, although *S*. Typhimurium var. Copenhagen with resistance to ampicillin, kanamycin, sulfamethoxazole, and tetracycline was a common finding in this study, the two herds with these bacteria produced less than 2% of the pigs marketed by study herds. This demonstrates why abattoir based monitoring, which can sample farms proportionate to the pork supply, can better estimate the food-safety risk from resistant *Salmonella*.

The primary weakness of this study was the small number of purposively selected herds. Selecting half of the herds with knowledge of the presumed *Salmonella* status did not appear to introduce a systematic bias, as the odds of resistance in these herds were not significantly different between herds of known- versus unknown- *Salmonella*-status. The other herd factor known at selection, herd size, was a significant predictor of resistance to streptomycin. By excluding herds with less than 100 sows, resistance to streptomycin might have been slightly overestimated. However, as most pigs in Saskatchewan and Alberta are raised on large farms, restricting this study to herds with more than 100 sows more realistically reflected the source of market pigs from these provinces (52). Additionally, this difference is clinically insignificant in comparison to the confidence estimates around the streptomycin resistance estimate.

Collecting both pooled and individual samples allowed us to address other research hypotheses. Identifying decreased odds of streptomycin resistance and increased odds of kanamycin resistance in *Salmonella* from individual-pig fecal samples compared to pooled pen samples is difficult to explain. An investigation of AMR in *E. coli* from cattle identified a non-significant trend towards less resistance in pooled, compared to individual, samples (53). Further investigation of this issue is needed because recently initiated Canadian and United States on-farm surveillance programs describe AMR from pooled fecal samples (16,38). Understanding the impact of sample type on resistance may influence the interpretation of these reports.

Using three isolation methodologies may have affected *Salmonella* recovery. However, the methods employed by two labs were very similar and this methodology was used on 89% of the samples. Culture methods can affect the serovars isolated, which could have introduced bias

into the data (35,54). Although this potential bias will continue to hinder efforts to compare AMR between labs until *Salmonella* isolation protocols are standardized, laboratory (and thus isolation procedure) was not a significant predictor of resistance to streptomycin, sulfamethoxazole or tetracycline. Although MIC testing occurred in two labs, 90% of the isolates were tested in one lab and the same MIC panels, methodology and quality control organisms were used by both labs to ensure comparability.

In summary, we described the resistance of *Salmonella* isolates from swine farms in Alberta and Saskatchewan. A high frequency of pansusceptibility, no resistance to six of the sixteen antimicrobials including ceftiofur and ciprofloxacin, and no resistance in the *Salmonella* Typhimurium DT 104 isolates are all encouraging findings. Even so, the extent of multiresistance in these isolates was concerning. Few on-farm studies have described AMR in *Salmonella* from all age categories of pigs (35,47). This project identified many future research needs. Age-specific risk factor studies are needed to investigate reasons for differences in resistance between production phases. Likewise, further description of the associations between resistance and serovar, and how resistance spreads within herds, are needed before effective intervention strategies can be designed to control AMR in *Salmonella* from pigs.

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Table 4.1 Frequency of the nine most common *Salmonella* serovars, the number of isolates per serovar with any resistance and number of isolates resistant to each antimicrobial (n = 468).

Salmonella Serovar (any resistance / all isolates)	Number of isolates from each serovar resistant to each antimicrobial									
	AMP	CEP	CHL	COT	FOX	GEN	KAN	STR	SMX	TET
Derby (56/124)	-	4	22	12	3	-	-	17	24	31
Typhimurium var. Copenhagen (65/81)	58	2	2	-	-	-	54	9	58	61
Putten (2/49)	-	-	1	1	-	-	-	1	2	2
Infantis (0/37)	-	-	-	-	-	-	-	-	-	-
I:ROUGH-O:d:l,w (3/34)	-	-	-	2	-	-	-	1	3	2
Banana (0/27)	-	-	-	-	-	-	-	-	-	-
Mbandaka (24/26)	1	-	-	-	-	-	-	19	12	24
Anatum (1/20)	-	-	-	-	-	-	-	1	1	1
Give (3/19)	-	-	1	-	-	-	-	-	3	-
All others (21/51)	3	-	5	3	-	1	1	7	11	17

a: AMP = ampicillin, CEP = cephalothin, CHL = chloramphenicol, COT = trimethoprim-sulfamethoxazole, FOX = cefoxitin, GEN = gentamicin, KAN = kanamycin, STR = streptomycin, SMX = sulfamethoxazole, TET = tetracycline

Table 4.2 Distribution of production phases from which nine most common serovars were isolated (n = 468).

Serovar	Total	Nursery	Grow-finish	Sow
Derby	124	6	83	35
Typhimurium var. Copenhagen	81	24	28	29
Putten	49	7	25	17
Infamtis	37	4	22	11
I:Rough-O:d:l,w	34	15	11	8
Banana	27	0	27	0
Mbandaka	26	14	12	
Anatum	20	3	9	8
Give	19	1	3	15
All Others	51	9	21	21

Table 4.3 Distribution of production phases from which 1394 samples were collected, 452 *Salmonella* positive samples were identified and 468 *Salmonella* isolates were harvested.

Production Phase	Sample Type	Samples Collected (%)	*Salmonella* Positive (%)	Isolates Harvested (%)
Nursery	PPS [a]	255 (18)	81 (18)	83 (18)
Grow-finish	Individual	295 (21)	73 (16)	80 (17)
	PPS	545 (39)	158 (35)	161 (34)
Sow	Individual	100 (7)	38 (38)	38 (8)
	PPS	199 (14)	102 (23)	106 (23)
Total		**1394**	**452**	**468**

a: PPS: pooled pen sample

Table 4.4 Percent of *Salmonella* isolates from nursery pigs (n = 83), grow-finish pigs (n = 241) and sows (n = 144) resistant to each drug.

	Percent resistant		
Antimicrobial	Nursery	Grow-finish	Sow
Tetracycline [b]	47.0	27.4	22.9
Sulfamethoxazole	41.0	16.6	27.8
Streptomycin [a,b]	22.9	8.7	10.4
Ampicillin [a]	19.3	9.1	16.7
Kanamycin [a]	16.9	7.9	15.3
Chloramphenicol	7.2	7.1	5.6
Trimethoprim-Sulfamethoxazole	2.4	3.7	4.9
Cephalothin [a]	2.4	0.8	1.4
Cefoxitin*	0.0	0.4	1.4
Gentamicin*	0.0	0.4	0.0

a: significant difference between isolates from nursery and grow-finish pigs ($P < 0.05$)
b: significant difference between isolates from nursery pigs and sows ($P < 0.05$)
c: significant difference between isolates from grow-finish pigs and sows ($P < 0.05$)
* models did not converge because frequency of resistance was too low

Table 4.5 Unconditional odds ratios for *Salmonella* pansusceptibility and multiresistance between isolates from each production phase (n = 468).

Outcome	Risk Factor Source of *Salmonella*	Odds ratio	95% confidence interval	*P*
Pansusceptible [a]	Sow compared with nursery	3.0	1.2 - 7.8	0.02
	Sow compared with grow-finish	2.3	1.1 - 4.6	0.02
	Grow-finish compared with nursery	1.3	0.7 - 2.5	0.37
Multiresistant [b]	Nursery compared with sow	3.2	1.1 - 9.1	0.03
	Nursery compared with grow-finish	2.6	1.0 - 7.0	0.05
	Grow-finish compared with sow	1.2	0.8 - 1.9	0.36

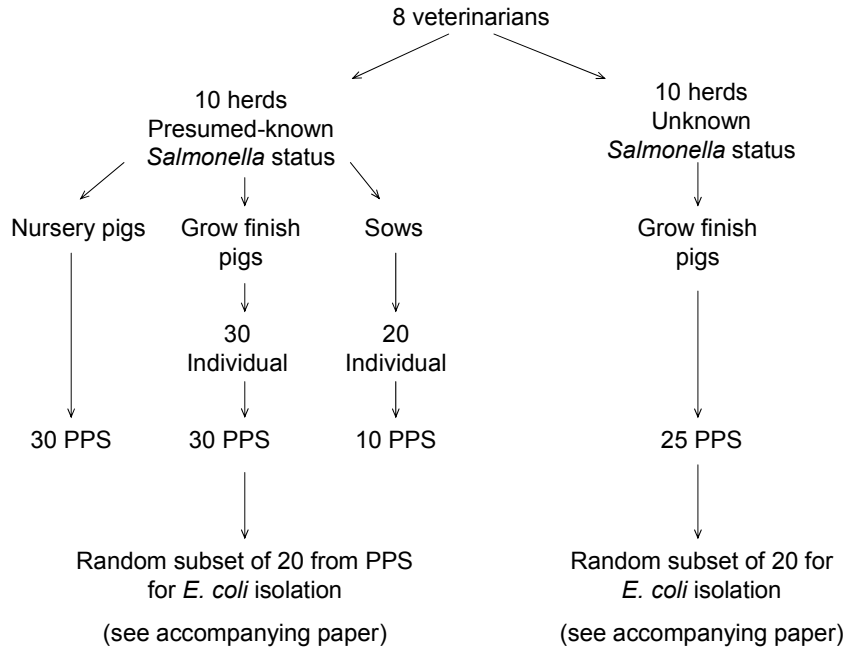
a: pansusceptible defined as susceptible to all sixteen drugs considered
b: multiresistant defined as resistant to two or more of the sixteen drugs considered

Table 4.6 *Salmonella* spp. resistance patterns observed in more than five isolates; number of isolates identified in each production phase, number of herds pattern was identified in, and number of serovars with pattern identified.

	Number of isolates			Number	Number
Resistance pattern	Nursery	Grow-finish	Sow	of Herds	of Serovars
AMP-KAN-SMX-TET	11	15	19	2	1
TET	1	31	4	4	6
SMX-STR-TET	5	4	5	4	3
STR-TET	6	8	0	2	3
CHL	0	6	1	3	2
CHL-SMX-STR-TET	5	2	0	1	5
AMP-KAN-SMX-STR-TET	2	1	3	1	1
SMX-TET	5	0	1	3	3
CHL-SMX-STR-COT	0	3	3	1	1

a: AMP = ampicillin, CHL = chloramphenicol, COT = trimethoprim-sulfamethoxazole, KAN = kanamycin, SMX = sulfamethoxazole, STR = streptomycin, TET = tetracycline

Figure 4.1 Schematic of herd and sample selection strategy.



PPS: pooled pen sample

Figure 4.2 Minimum inhibitory concentration distributions, population average prevalence estimates, and 95% confidence intervals for resistance to each drug in *Salmonella* isolates (n = 468).

			Distribution of Isolates (Count) Across Dilution Ranges																
Antimicrobial	Prevalence	95% CI	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Tetracycline	34.5	19.4 - 53.6									**329**	1			138				
Sulfamethoxazole	26.7	13.3 - 46.3											36	80	**193**	45		1	113
Streptomycin	13.6	7.5 - 23.3												**413**	27	28			
Ampicillin	12.6	3.4 - 36.7							**263**	60	72	5	6	1	61				
Kanamycin	10.2	2.6 - 33.0										**413**				55			
Chloramphenicol	5.0	1.6 - 14.3								1	60	**265**	111	11	20				
Trimethoprim-Sulfamethoxazole	2.7	0.6 - 11.2				**363**	83	1		3	2	16							
Cephalothin	1.2	0.5 - 3.4								134	**208**	83	37	6					
Cefoxitin	0.6	0.2 - 2.2							2	137	**207**	45	74	3					
Gentamicin	0.2	0.0 - 1.7					**382**	48	37				1						
Amikacin	0	0.0 - 0.6						73	**316**	73	5	1							
Amoxicillin-Clavulanic Acid	0	0.0 - 0.6							**315**	90	2	54	7						
Ceftiofur	0	0.0 - 0.6					1	191	**200**	76									
Ceftriaxone	0	0.0 - 0.6					**467**			1									
Ciprofloxacin	0	0.0 - 0.6	**379**	50	38	1													
Nalidixic Acid	0	0.0 - 0.6								41	**343**	81	3						

Shaded fields indicate the range tested for each antimicrobial. Counts outside of shaded cells indicate isolates with growth in all wells within the tested range. For these isolates the actual MIC is greater than that range of dilutions. Vertical double bars mark the breakpoint between sensitive and resistant. Bold font indicates the median MIC.

CHAPTER 5
ANTIMICROBIAL RESISTANCE OF FECAL *ESCHERICHIA COLI* ISOLATED FROM GROW-FINISH PIGS IN 20 HERDS IN ALBERTA AND SASKATCHEWAN, CANADA

5.1 Introduction

Antimicrobial resistance (AMR) in bacteria from food animals is a worldwide public health issue. Zoonotic infections and the transmission of resistance genes to commensal and pathogenic bacteria of humans are frequently cited concerns (1-3). The frequency of AMR in commensal organisms reflects the selective pressure exerted on bacteria to develop resistance and the potential reservoir of resistance genes available for dissemination to pathogens (2,4). For these reasons, the World Health Organization (WHO) and World Organization for Animal Health (OIÉ) recommend monitoring AMR in commensal organisms including *E. coli* (5,6). *Escherichia coli* are highly prevalent in healthy animals, facilitating comparisons of AMR within and between species (2,7,8).

Pork is among the three most commonly consumed animal proteins in Canada and is the most common worldwide (9,10). The frequency of resistance in *E. coli* from pigs sampled at Canadian abattoirs, and pork sampled at retail, has been intermediate between chicken and beef (7). This resistance, combined with the extensive use of antimicrobials in pork production, (11-13), has created interest in antimicrobial resistant bacteria in pigs.

Previous Canadian studies have considered *E. coli* from swine farms in Ontario and British Columbia, and on-farm surveillance has been initiated (7,14,15). This study

investigated AMR in *E. coli* from apparently healthy grow-finish pigs on farms in Alberta and Saskatchewan, Canada. Three analyses described the resistance in these isolates. First, unconditional associations between resistance phenotypes described the potential for co-selection in these isolates. Second, *E. coli* was evaluated as a farm-level sentinel for AMR in *Salmonella*. Finally, others have observed that resistance clusters within pigs, pens and herds (16-19); this project quantified the variation in resistance between isolates, within herds, to determine the value of investigating farm-level risk factors for AMR.

5.2 Materials and Methods

5.2.1 Herd and sample selection

A convenience sample of 20 farms was allocated to eight swine veterinarians in Saskatchewan (13 farms) and Alberta (7 farms). Farms were selected by veterinarians based on a minimum herd size of 100 sows and enrollment with the Canadian Quality Assurance® (CQA) Program (20). The number of farms per veterinarian ranged from two to four. Half of the veterinarians were asked to identify the presumed *Salmonella* status of the herds and 10 herds were enrolled with knowledge of the presumed *Salmonella* status (see accompanying *Salmonella* paper). Each herd was sampled once between May and September of 2004. Fecal samples were collected from 20 randomly selected pens per herd. Samples were pooled at the pen level and each consisted of feces from five grow-finish pigs.

5.2.2 Laboratory methods

Samples were manually mixed, shipped on ice to a commercial veterinary laboratory (Prairie Diagnostic Services (PDS), University of Saskatchewan, Saskatoon, SK) and cultured for *E. coli* within 24 h of collection. Each sample was streaked onto a whole Blood Agar and MacConkey plate with a heavily coated cotton swab and incubated at 37° C for 18 h. Three lactose-positive colonies were selected from each MacConkey plate unless distinct colonies, such as hemolytic and non-hemolytic or mucoid and dry were identified; up to six were harvested from those samples. Selected colonies were incubated in trypticase soy (TS) broth for 3 to 4 h at 37° C. Each TS broth was inoculated onto urea and citrate slants with a 1 µl loop, and a blood agar plate was concurrently inoculated to ensure sample purity. These were incubated at 37° C for 18 h. Samples requiring further confirmation were tested with Triple Sugar Iron / Indole. Pure, confirmed *E. coli* cultures were frozen in 25% glycerol at -80° C until tested for antimicrobial susceptibility.

Antimicrobial susceptibility testing was conducted by the Agri-Food Laboratories Branch (AFLB), Food Safety Division of Alberta Agriculture, Food and Rural Development, Edmonton, AB and PDS, Saskatoon, SK. Isolates were tested using a broth microdilution technique following Clinical and Laboratory Standards Institute (CLSI) guidelines (21,22). National Antimicrobial Resistance Monitoring System (NARMS) minimum inhibitory concentration (MIC) CMV7CNCD susceptibility panels (Sensititre^{TM}, TREK Diagnostic Systems, Westlake, Ohio) were used to test isolates for susceptibility to 16 antimicrobials across a standard range of dilutions (Figure 5.1). Each isolate was grown up on a non-selective media. A 0.5 McFarland standard was made in 5

ml of demineralized water of which 10 µl was transferred into 11 ml of cation-adjusted Mueller-Hinton broth with TES buffer. A 50 µl aliquot was inoculated into each of the 96 wells on the panel. Inoculated plates were incubated and read by the Sensititre ARIS® (Automated Reading and Incubation System) (TREK Diagnostic Systems, Inc., Westlake, OH). Readings were transferred to Sensititre Automated Microbiology Systems (SAMS) computer software (TREK Diagnostic Systems) and interpreted according to CLSI breakpoints for animals or humans (21,22) (Figure 5.1). The MIC breakpoint for streptomycin, which does not have a CLSI guideline, was taken from the National Antimicrobial Resistance Monitoring System (NARMS) 2000 *E. coli* report (23). Quality control organisms used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeroginosa* ATCC 27853.

5.2.3 Data comparisons and statistical analysis

Intermediate MIC values were categorized as susceptible (21,22). Descriptive analyses were calculated with commercially available software (Microsoft Excel, Microsoft Corporation, Redmond, Washington, USA) and statistical analyses were performed using generalized estimating equations (GEE) (PROC GENMOD, SAS version 9.1, SAS Institute Inc., Cary, North Carolina) to adjust for clustering of resistance within herds. All models had a logit-link function, binomial distribution and exchangeable correlation structure. Unless stated otherwise, the outcome variable was 'any resistance' to a given drug versus 'no resistance' (dichotomous) at the isolate level. The association between each variable of interest and outcome was reported as an odds ratio (OR = expβ) with 95% confidence intervals (CI) (24).

The population average prevalence for resistance to each antimicrobial was calculated using the intercept (β_0) and 95% CI from a null binomial response model in $[1 + \exp(-\beta_0)]^{-1}$(24). Beyond these prevalence estimates, analyses were restricted to drugs with more than five percent prevalence of resistance to minimize problems with power, model stability and convergence.

In the first set of analyses, the associations between different resistance phenotypes were considered. As there were seven drugs with more than five percent prevalence of resistance, each outcome was unconditionally considered against six other drug-resistances. Based on this, the cut off for statistical significance was adjusted with a Bonferroni correction and reported significant if $P < 0.007$ (0.05/7) (24).

The second set of analyses explored potential biases introduced by study design. The unconditional associations between *E. coli* resistance phenotypes and two herd characteristics known at selection, herd size and knowledge of the presumed-herd-*Salmonella* status, were evaluated. These associations were reported significant if $P < 0.05$.

The associations between resistances observed in *Salmonella* spp. and *E. coli* were measured to assess the potential for herd-level *E. coli* AMR data to predict resistance in *Salmonella*. Susceptibility data were generated concurrently in these herds (Chapter 4). The outcome variables represented *Salmonella* resistance to each drug, and

were modeled as the number of resistant isolates in the herd as the numerator and the number of isolates tested in each herd as the denominator. The predictor variable was the proportion of *E. coli* in the herd that were resistant to the same drug. These associations were reported significant if $P < 0.05$.

A different approach to modeling AMR estimated the extent that resistance clustered within herds, and within the veterinarians servicing the herds. The variance at each level was determined using restricted maximum likelihood estimation. The resistance phenotype outcome was modeled as the proportion of resistant isolates in the herd as the numerator and the number of isolates tested in each herd as the denominator. The restricted generalized iterative least-squares (RIGLS) algorithm in MLwiN (MLwiN version 2.0r, Centre for Multilevel Modelling, Institute of Education, University of London, London, England) was used and second-order penalized quasi-likelihood (PQL-2) estimates were reported. Under-dispersion was accounted from by allowing random variation at the lowest level (25). Non-significant hierarchical levels, based on the liberal criteria of the standard error being larger than the variance estimate, were excluded from further consideration. The intra-class correlation (ICC) between isolates within herds was approximated by the latent variable approach (24,25). Specifically, the herd variance was divided by the total variance after fixing the error variance at $\pi^2/3$.

5.3 Results

Four hundred and five fecal samples were cultured and 1439 *E. coli* harvested for an average of 63 isolates per herd (range, 60 to 88). The prevalence of resistance was highest to tetracycline, sulfamethoxazole and streptomycin, while no resistance was

observed to ceftriaxone or ciprofloxacin (Figure 5.1). For amikacin, six isolates had an MIC greater than the dilution range tested. Because the breakpoint was also above the highest dilution, the status of these isolates was indeterminable.

Twenty-one percent of the *E. coli* were susceptible to all drugs on the test panel while 57.0% (95% CI, 47.2 to 66.2) were resistant to two or more (Table 5.1). Two isolates were resistant to nine antimicrobials. Ninety-two unique resistance patterns were identified. Combinations of resistance to tetracycline, sulfamethoxazole and streptomycin accounted for the four most common patterns, and these antimicrobials were involved in all of the ten most common resistance patterns (Table 5.2).

In these *E. coli*, resistance to each drug was significantly associated with resistance to at least two other drugs. The odds of an isolate being resistant to sulfamethoxazole increased significantly if it was resistant to any other drug considered (Table 5.3). Because only one isolate was both resistant to trimethoprim-sulfamethoxazole and susceptible to sulfamethoxazole, the associations between these resistances were very strong. The next strongest associations were between resistance to sulfamethoxazole and chloramphenicol.

The odds of an isolate being resistant to streptomycin, sulfamethoxazole and tetracycline each decreased by 0.99 times for every 1000 pigs finished annually ($P = <$ 0.0001, 0.007 and 0.049 respectively). *E. coli* resistance to ampicillin, chloramphenicol, kanamycin and trimethoprim-sulfamethoxazole were not significantly associated with

herd size ($P > 0.23$). No resistance was significantly associated with knowledge of the presumed *Salmonella* status ($P > 0.09$).

The frequency of *E. coli* resistance in the herd was a significant predictor of *Salmonella* resistance to two drugs. For each one percent increase in *E. coli* resistance to kanamycin the odds of *Salmonella* resistance to kanamycin increased 1.24 times (95% CI, 1.15 to 1.34; $P = 0.001$). Similarly, for trimethoprim-sulfamethoxazole, a one percent increase in *E. coli* resistance increased the odds of *Salmonella* resistance by 1.32 times (95% CI, 1.10 to 1.58, $P = 0.003$). The associations for the remaining five drugs were not significant ($P > 0.19$).

For all resistance phenotypes, the variance at the veterinary practitioner level was not significant ($P > 0.31$) while the variance at the herd level was highly significant ($P = 0.003$ to 0.017). Hence, variance at the veterinary practitioner level was not considered when calculating the ICCs. The ICC between isolates within herds was smallest for streptomycin and largest for kanamycin (Table 5.4).

5.4 Discussion

Antimicrobial resistance in *E. coli* from pigs has been well described in North America. Nationally representative, abattoir-based monitoring in Canada and the United States is ongoing, an on-farm monitoring program completed its pilot phase in the United States, and a similar Canadian program is beginning its second year (7,23,26). Targeted cross-sectional studies in Ontario and British Columbia have described *E. coli* AMR on Canadian swine farms (14,15). This study differed from these previous reports by

describing *E. coli* from swine farms in Alberta and Saskatchewan, Canada. Identifying substantial regional-level variation in AMR could prompt investigation into differences in management or antimicrobial use between provinces. This study also considered the ability of *E. coli* to predict *Salmonella* AMR within herds and the extent resistance clustered within herds.

The frequency of resistance to at least one drug, and to each individual drug, was comparable to other North American reports (7,14,23,26). Therefore, despite describing resistance in a small number of herds, our findings appear relevant to other regions in North America. Slight differences between these findings and previous reports were a lower prevalence of resistance to ampicillin (19 versus 22 to 35%) and a higher prevalence to chloramphenicol (17 versus 8 to 13%) (7,14,26).

Resistance to chloramphenicol was particularly interesting because this drug was banned for use in Canadian food animals in 1985 (27). Florfenicol was not used in study herds in the 12 months prior to sample collection (Chapter 3). Therefore, direct selection for the *floR* gene, which confers resistance to both florfenicol and chloramphenicol, is unlikely (28). Rather, chloramphenicol resistance likely persisted due to co-selection. Co-selection occurs with transmission of linked AMR genes on plasmids, transposons, and integrons. Bacteria resistant to multiple drugs have a competitive advantage in a wider range of environments (4,29,30). The strong associations between chloramphenicol and sulfamethoxazole resistance suggests co-selection may be occurring between resistance genes encoding for these drugs. This hypothesis is supported by reports of co-

transmission of chloramphenicol and sulfonamide genes on transmissible plasmids, and significant odds ratios between genes encoding for resistance to these drugs (28,31). However, further molecular study of these isolates is required to confirm this hypothesis.

In addition to being associated with chloramphenicol, sulfamethoxazole resistance was associated with every other drug resistance. The *sul1* gene, which encodes for sulfamethoxazole resistance, is a component of class I integrons, genetic elements that acquire and link resistance gene cassettes (29). Thus, integrons carrying resistance genes could explain the associations between sulfamethoxazole and other drug resistances. Sulfonamide use has been associated with sulfamethoxazole resistance (14). By extension, our findings suggest it could be influencing resistance to many other antimicrobials. This is important considering sulfonamides are commonly administered to Canadian pigs (12,13). Overall, identifying numerous associations between resistances means co-selection between unrelated antimicrobials must be considered when making drug use decisions.

Antimicrobial resistant *Salmonella* are a more obvious food safety hazard than *E. coli,* given that outbreaks of resistant salmonellosis in human have been linked to consuming contaminated pork (32,33). However, monitoring *Salmonella* AMR is challenging because sub-clinically infected pigs shed intermittently, barns fluctuate between a *Salmonella*-positive and apparent-negative status, and isolation techniques can have poor sensitivity (34,35). In contrast, *E. coli,* another member of the Enterobacteriaceae family, is highly prevalent and easily isolated. *Salmonella* and *E. coli*

from pigs can share resistance genes in vitro and molecular evidence suggests transmission occurs in vivo (36,37). Despite this, herd-level resistance in *E. coli* was not a predictor of resistance in *Salmonella* for five of seven phenotypes. Although this appears inconsistent with using *E. coli* as indicator bacteria, the lack of associations may be due to study design. There was a relatively low number of *Salmonella* isolates in some herds, which precluded considering serovar as a confounding variable. Additionally, the relationship was described at the herd, rather than pen, level. Our findings could be also due to describing resistance by phenotype rather than genotype or clonal resistance in *Salmonella* within herds. These factors could easily overshadow any shared resistance genes between *E. coli* and *Salmonella* within herds.

Swine barns have a hierarchical structure; pigs are grouped in pens, pens in rooms, rooms in barns, and barns in production companies. The variation in resistance between each of these levels, as well as between pigs at different time points, has been estimated (15-17,38). Variation between herds (operated by different people or production companies) has been described (15,18,19). Describing the extent that resistance varies within herds is valuable because many management decisions, including antimicrobial use, are made at this level. Identifying substantial variation suggests these practices may influence resistance (24). The intra-class correlations between isolates within farms, which were similar to those reported by Dunlop et al., demonstrated that on-farm risk factor studies for resistances should be undertaken, and statistical analyses should account for the lack of independence in AMR data (15,24). In contrast, the lack of variation between veterinary practitioners indicated that interventions targeted at

veterinary practitioners would likely have a negligible impact on resistance compared to those targeted at farms.

Comparisons with other AMR data should be made cautiously because selection criteria, sampling strategy and laboratory methods may influence results. Antimicrobial susceptibility testing methods were chosen to allow direct comparison with existing national surveillance data from Canada and the United States (7,23,26). Antimicrobial resistance in *E. coli* could vary with geographical, temporal or management factors: Despite this our findings were similar to AMR data from other North American farms and Canadian abattoirs (7,14,15,23,26). This suggests data from regional studies is useful for understanding AMR in *E. coli* from healthy pigs in North America.

Exploring herd characteristics influenced by the selection strategy found no significant associations between any resistance and knowledge of the herd S*almonella*-status. This indicates that participation bias, due to the more intensive sampling in known-*Salmonella*-status herds, was unlikely. In contrast, herd size was significantly associated with three resistance outcomes. For these drug-resistances, limiting our study to herds with more than 100 sows may have resulted in slightly less observed resistance than if all herds had been eligible. However, although 70% of Saskatchewan pig farms market less than 1000 pigs annually (which roughly corresponds to herds of 50 sows or less) they produce less than 3% of Saskatchewan's pigs (39). Therefore, restricting the study to larger herds improved study representation of market hog production in western Canada. Although this study utilized a convenience sample of herds, the investigators had

no knowledge at selection of the antimicrobial use patterns in the herds. This was presumed to be the primary influence on resistance. Participation in this study was restricted to herds enrolled in the CQA® program to ensure adequate antimicrobial use records for other aspects of the study (20). Although participation in the CQA® program may influence antimicrobial use practices, it is unlikely to have biased results as more than 98% of production units in Alberta and Saskatchewan participate in this program (personal communication Sarah Turner, Alberta Pork and Harvey Wagner, Sask Pork).

The findings of this study reflect the on-farm selection pressure for AMR and the potential food-safety risk from near-market animals. Describing *E. coli* AMR in Alberta and Saskatchewan herds provides baseline information for monitoring on-farm AMR in *E. coli.* With further study on swine farms, there is potential to identify risk-factors for antimicrobial resistance.

5.5 References

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Table 5.1 Frequency, prevalence and 95% confidence intervals of number of antimicrobials in *E. coli* antimicrobial resistance pattern (n = 1439).

Drugs in resistance pattern	Frequency	Prevalence	95% Confidence Interval
None	298	20.9	15.0 - 28.3
One	320	22.2	18.3 - 26.7
Two	269	18.6	15.7 - 21.8
Three	263	18.3	14.5 - 22.7
Four	186	12.8	9.4 - 17.3
Five	75	5.4	3.4 - 8.4
Six	23	1.6	0.9 - 2.7
Seven	3	0.2	0.1 - 0.8
Eight	0	0.0	0.0 - 0.2
Nine	2	0.1	0.0 - 0.5

Table 5.2 Ten most common antimicrobial resistance patterns observed in *E. coli* (n = 1439).

AMR Pattern [a]	Frequency	Percent
TET	235	16.3
SMX-TET	98	6.8
STR-SMX-TET	97	6.7
STR-TET	94	6.5
CHL-STR-SMX-TET	56	3.9
CHL-SMX-TET	45	3.1
STR	36	2.5
SMX	35	2.4
AMP-STR-TET	34	2.4
AMP-TET	31	2.2

a: AMP: ampicillin; CHL: chloramphenicol; SMX: sulfamethoxazole; STR: streptomycin; TET: tetracycline

Table 5.3 Significant univariate associations between antimicrobial resistances ($P <$ 0.007), (n = 1439).

Variable Outcome	Predictor	Odds Ratio	95% Confidence Interval	*P*
Ampicillin	Kanamycin	3.1	1.6 - 6.0	0.001
	Streptomycin	2.0	1.2 - 3.1	0.004
	Tetracycline	2.5	1.8 - 3.5	≤0.001
	Sulfamethoxazole	2.3	1.4 - 3.7	≤0.001
	Trimethoprim-sulfamethoxazole	5.7	2.6 - 12.6	≤0.001
Chloramphenicol	Sulfamethoxazole	34.6	12.6 - 95	≤0.001
	Trimethoprim-sulfamethoxazole	3.3	1.7 - 6.4	≤0.001
Kanamycin	Ampicillin	2.8	1.5 - 5.2	0.001
	Streptomycin	4.2	1.9 - 8.9	≤0.001
	Sulfamethoxazole	5.9	3.2 - 10.9	≤0.001
	Trimethoprim-sulfamethoxazole	1.4	1.1 - 1.7	0.005
Streptomycin	Ampicillin	2.1	1.3 - 3.4	0.002
	Kanamycin	5.2	2.1 - 12.5	≤0.001
	Tetracycline	5.6	3.9 - 7.9	≤0.001
	Sulfamethoxazole	2.7	1.9 - 3.8	≤0.001
Sulfamethoxazole	Ampicillin	2.2	1.4 - 3.5	0.001
	Kanamycin	5.6	2.7 - 11.5	≤0.001
	Streptomycin	2.4	1.8 - 3.2	≤0.001
	Tetracycline	2.9	1.9 - 4.5	≤0.001
	Trimethoprim-sulfamethoxazole	118	14 - 968	≤0.001
	Chloramphenicol	31	13 - 73	≤0.001
Tetracycline	Ampicillin	2.3	1.7 - 3.2	≤0.001
	Streptomycin	4.6	3.3 - 6.5	≤0.001
	Sulfamethoxazole	3.1	2.1 - 4.7	≤0.001
Trimethoprim-sulfamethoxazole	Ampicillin	6.5	3.0 - 14.2	≤0.001
	Sulfamethoxazole	215	9.0 - 5170	≤0.001
	Chloramphenicol	3.6	1.8 - 7.2	≤0.001

Table 5.4 Distribution of within herd prevalence of resistance, variance attributed to clustering within herds and intra-class correlation between isolates within herds (N = 20 herds; n = 1439 *E. coli*).

	Within Herd prevalence		Herd Variance	
Antimicrobial	Median	IQR	(standard error)	ICC[a]
Ampicillin	14.2	11.1 - 27.6	1.0 (0.35)	0.23
Chloramphenicol	10.5	4.2 - 20.9	2.0 (0.71)	0.37
Kanamycin	4.6	0.9 - 11.2	2.8 (1.1)	0.46
Streptomycin	34.0	22.5 - 43.2	0.4 (0.14)	0.10
Sulfamethoxazole	42.3	28.6 - 64.7	1.2 (0.39)	0.26
Tetracycline	62.1	49.9 - 82.0	1.4 (0.48)	0.30
Trimethoprim-Sulfamethoxazole	8.1	1.4 - 11.3	1.1 (0.04)	0.24

a: ICC = Intra-class correlation coefficient between isolates within herd

Figure 5.1 Minimum inhibitory concentration distributions, population average prevalence estimates, and 95% confidence intervals for resistance to each drug in *E. coli* isolates (n = 1439).

	Prevalence of		Distribution of Isolates (Count) Across Dilution Range																
Antimicrobial	resistance	95% CI	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Tetracycline	66.8	57.9 - 74.7									452	21	23	98	**845**				
Sulfamethoxazole	46.0	36.4 - 56.0											**721**	42	11	4		4	657
Streptomycin	33.4	27.8 - 39.5												**961**	256	222			
Ampicillin	18.6	17.9 - 19.2							138	**615**	381	33	6	2	264				
Chloramphenicol	17.3	10.7 - 26.7								76	601	**453**	60	117	132				
Kanamycin	9.3	4.6 - 18.0										**1299**	7	2	3	128			
Trimethoprim-Sulfamethoxazole	7.4	5.1 - 10.5				**890**	333	78	21	11	1	105							
Cephalothin	3.8	2.2 - 6.6								55	382	**665**	282	42	13				
Gentamicin	0.8	0.4 - 1.8					315	**621**	476	8	1	6	6	6					
Cefoxitin	0.6	0.2 - 1.6						2	24	406	**767**	223	8	9					
Amoxicillin-Clavulanic Acid	0.4	0.1 - 1.3							88	508	**609**	206	22	4	2				
Ceftiofur	0.1	0.0 - 0.5				175	**1002**	249	6	3	3	1							
Nalidixic Acid	0.1	0.0 - 0.5						10	135	**1037**	246	10			1				
Amikacin	0	0.0 - 0.2						68	594	**664**	107	6							
Ceftriaxone	0	0.0 - 0.2					**1427**	4	6	2									
Ciprofloxacin	0	0.0 - 0.2	**1426**	10	1	1		1											

Shaded fields indicate the range tested for each antimicrobial. Counts outside of shaded cells indicate isolates with growth in all wells within the tested range. For these isolates, the actual MIC is greater than that range of dilutions. Vertical double bars mark the breakpoint between sensitive and resistant. Bond font indicates the median.

CHAPTER 6
ANTIMICROBIAL RESISTANCE AND VIRULENCE GENES OF COMMENSAL *ESCHERICHIA COLI* FROM GROW-FINISH PIGS

6.1 Introduction

Antimicrobial resistance (AMR) in *Escherichia coli* is a serious concern in human and veterinary medicine. Resistant infections are associated with increased morbidity, mortality and treatment expense compared to their susceptible counterparts (1-4). Resistant commensal *E. coli*, while not causing direct disease, are a reservoir of antimicrobial resistance genes. These genes can be transferred to zoonotic pathogens, such as *Salmonella*, or to other gram-negative bacteria in the gut (5,6). Pathogenic and commensal strains of *E. coli* have different rates of resistance and carry different genes (7,8). Therefore, describing the antimicrobial resistance genes in commensal *E. coli* documents the diversity of genes available for dissemination to other bacteria.

In a previous paper, we described the frequency and patterns of phenotypic AMR in *E. coli* from healthy grow-finish pigs in western Canada. Of 1439 isolates, 21% were susceptible to all of the sixteen drugs considered while 57% were resistant to two or more antimicrobials (Chapter 5). This paper describes the presence of resistance and virulence genes in a subset of those isolates. The data were described through three sets of analyses. First, unconditional associations between resistance genes were assessed; these associations generate hypotheses about the physical relationships between genes that dictate co-selection. Secondly, associations among resistance phenotypes were analyzed

and compared to the associations among resistance genes. Identifying similar relationships in these analyses would suggest that phenotypic resistance data could also generate hypotheses about co-selection. Finally, associations between resistance and virulence genes were investigated. Others have reported more frequent resistance, and numerous associations between resistance and virulence genes, in pathogenic *E. coli* (7,8). This has created concerns that antimicrobial exposure contributes to the persistence and spread of virulence in *E. coli* (4,9,10). Describing similar associations in commensal *E. coli* would indicate a potential for antimicrobial use to increase virulence in *E. coli* carried by healthy pigs.

6.2 Materials and Methods

6.2.1 Herd and sample selection

Twenty herds were enrolled in the study by eight swine veterinarians in Saskatchewan (thirteen herds) and Alberta (seven herds). Herds were selected by each veterinarian to match the study inclusion criteria of a minimum of 100 sows and enrollment in the Canadian Quality Assurance® Program (11). The number of herds per veterinarian ranged from two to four. Each herd was visited once between May and September of 2004. Twenty pens of apparently healthy, grow-finish pigs were randomly identified in each herd. Freshly voided fecal samples were collected from five pigs in each of these pens, and then pooled to create a pen-level sample.

6.2.2 Culture and antimicrobial susceptibility testing

Samples were manually mixed, shipped on ice to a commercial veterinary laboratory (Prairie Diagnostic Services (PDS), University of Saskatchewan, Saskatoon,

SK) and cultured for *E. coli* within 24 h of collection. Each sample was streaked onto whole Blood Agar and MacConkey plates then incubated at 37° C for 18 h. Three lactose-positive colonies were selected from each MacConkey plate unless distinct colonies, such as hemolytic and non-hemolytic or mucoid and dry were identified; up to six were harvested from those samples. Pure, confirmed *E. coli* cultures were stored in 25% glycerol at -80° C for susceptibility testing.

Antimicrobial susceptibility testing was conducted by the Agri-Food Laboratories Branch, Food Safety Division of Alberta Agriculture, Food and Rural Development, Edmonton, AB and PDS, Saskatoon, SK. Isolates were tested for antimicrobial susceptibility using a broth micro-dilution technique following Clinical and Laboratory Standards Institute (CLSI) guidelines. National Antimicrobial Resistance Monitoring System (NARMS) minimum inhibitory concentration (MIC) CMV7CNCD susceptibility panels (Sensititre™, TREK Diagnostic Systems, Westlake, Ohio) were used to test isolates for susceptibility to 16 antimicrobials across a standard range of dilutions (12,13). Each isolate was grown up on a non-selective media. A 0.5 McFarland standard was made in 5 ml of demineralized water, of which 10 µl was transferred into 11 ml of cation-adjusted Mueller-Hinton broth with TES buffer. A 50 µl aliquot was inoculated into each of the 96 wells on the panel. Inoculated plates were incubated and read by the Sensititre ARIS® (Automated Reading and Incubation System) (TREK Diagnostic Systems, Inc., Westlake, OH). Readings were transferred to Sensititre Automated Microbiology Systems (SAMS) computer software (TREK Diagnostic Systems) and interpreted according to CLSI breakpoints for animals or humans (12,13). Streptomycin

does not have a CLSI MIC breakpoint, therefore this breakpoint was taken from the National Antimicrobial Resistance Monitoring System (NARMS) 2000 *E. coli* report (14). Quality control organisms used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeroginosa* ATCC 27853.

6.2.3 Isolate selection

One hundred and fifty-one *E. coli* were selected from the 1439 available isolates. Isolate selection was purposive to ensure a minimum of 15 isolates were resistant to each of the following antimicrobials: ampicillin, chloramphenicol, kanamycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim-sulfamethoxazole. More than 5% of the 1439 available *E. coli* were resistant to these antimicrobials (Chapter 3). Only these seven resistance phenotypes and genes encoding for resistance to these seven drugs were considered in this study. Clustering was minimized by selecting a maximum of one isolate per pen while maintaining the original herd distribution.

6.2.4 Antimicrobial resistance gene detection

AMR gene testing was performed by the Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec. The 28 strains used as positive controls and templates for DNA amplification were obtained from different laboratories (Table 6.1) (2,15). These strains were maintained as frozen stocks at -80° C in tryptic soy broth medium containing 10% glycerol (vol/vol). They were propagated on Luria-Bertani broth or agar containing one of the following antimicrobial agents at the appropriate concentrations: ampicillin (50

μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), trimethoprim (10 μg/ml), and sulfamethazine (200 μg/ml).

The primers for *cmlA*, *strA*, *strB* and *sul3* had been previously described and validated (7,8). Resistance gene primers were designed by using the software program Prime (Genetics Computer Group, Madison, Wis.). Oligonucleotide primers were synthesized with a DNA synthesizer (BioCorp Inc., Montreal, Quebec, Canada). The PCR primers, their amplified product sizes and the references for the corresponding strains used as amplification templates have been described by Maynard et al. (Table 6.1) (2,15).

Template DNA was prepared from bacterial cultures by the boiling method of Daigle et al. (16). Amplifications were performed with 5 μl of supernatant from bacterial preparations that had been boiled for 10 m (16). The PCR mixture (total volume, 50 μl) included 29.6 μl of H_2O, 5.0 μl of 10x PCR buffer (Amersham Pharmacia Biotech Inc., Piscataway, N.J.), 2 μM each of the four deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Amersham Pharmacia Biotech Inc.), 25 pmol of each primer and 5 μL of template. DNA amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, Calif.) by using the following conditions: 5 m at 94° C, followed by 30 cycles of 94° C for 30 s, 50° C for 30 s, and 72° C for 1.5 m. A 3 μl aliquot of the PCR product was verified for size and purity by gel electrophoresis (1.2% [wt/vol] agarose in 1x TAE [Tris-acetate-EDTA] buffer). The amplicons were labeled with [α-^{32}P] dCTP by using a DNA labeling beads kit (Amersham Pharmacia Biotech Inc.). Colony hybridizations were performed as described previously (17).

6.2.5 Virulence gene detection

Virulence gene testing was conducted by PDS, Saskatoon, SK. Each strain was grown up on blood agar plates and two to four *E. coli* colonies were randomly selected and mixed in 400 µl of D-Solution (4 M GuSCN, 25mM Na citrate at Ph 8.0, 0.5% sarcosyl, 0.1 M βME (700 µl in 100 ml)) (Sigma, Oakville, ON). To each tube, 100µl of TE-saturated phenol (Sigma) and 100 µl of chloroform (Sigma) were added and the tube was mixed. The DNA was lysed by incubation at -20° C for 10 m and then centrifuged for 5 m at 4° C and 15,000 g and the aqueous layer removed. The process of washing, mixing and centrifuging were repeated at least once, or until the interface was clear. Five hundred microlitres of 95% salted ethanol (VWR, Luterworth, Leicestershire, England) was added, inverted to mix, and incubated for 1 to 12 h at -20° C. The tube was then centrifuged for 15 m at 4 ° C at 15,000 g and the ethanol decanted off. The DNA pellet was dried for 5 to 10 m at 30 to 35 ° C and dissolved in 80 to 100 µl of sterile water.

Oligonucleotide primers were used for the detection of virulence associated genes (Table 6.2). Bacterial DNA amplification was performed in 30 µl of sterile, ultra-pure water, 5 µl 10x PCR buffer, 4 µl (25 mM/µl) $MgCl_2$, 0.5 µl dNTPs (25 mM/µl), 0.5 µl *Taq* polymerase (5 U/µl) and four primers (2 µl per primer, 20 pmol/µl) (Fermentas, Burlington, ON). Two microlitres of DNA were dispensed into each tube, centrifuged for 30 s and then immediately placed into the preheated cycler. The cycler denatured the DNA for 2 m at 94° C then amplified it by 35 cycles of the following: denaturing for 30 s at 94° C, annealing of primers for 30 s at 60° C and extension for 30 s at 72° C. Final extension occurred for 10 m at 72° C and the reaction was concluded at 10° C. The PCR amplicons were visualized following electrophoresis on 1.25% agarose gel and staining

with ethidium bromide. Amplicons were compared to a sterile negative control and a positive reference strain (Table 6.2). Results were recorded with an Alpha Imager documentation camera (Fisher Scientific, Ottawa, Ontario).

6.2.6 Statistical analyses

Minimum inhibitory concentration, AMR gene, and virulence factor data were maintained in a relational database (Microsoft Access, Microsoft Corporation, Redmond, Washington, USA). For the purpose of this analysis, isolates with intermediate MICs were considered susceptible. Descriptive and non-parametric statistics were performed with commercially available software (SPSS version 14.0 for Windows, Chicago, IL, USA) while tests of association were conducted using generalized estimating equations (GEE) (PROC GENMOD, SAS version 9.1 for Windows; SAS Institute, Cary, North Carolina, USA). Model outcomes were at the isolate level and adjusted for clustering of resistance within herds.

The association between the number of drugs in each isolate's resistance phenotype (outcome) and number of resistance genes detected (predictor) was investigated using a Poisson distribution, a log link function, and an exchangeable correlation structure. Associations were considered statistically significant if $P < 0.05$ and were reported as a risk ratio (RR = expβ) with 95% confidence intervals (CI).

Resistance to each drug was described in two ways: i) an isolate had a resistant phenotype if the MIC was greater than the breakpoint; and ii) an isolate had a resistant genotype if it carried a gene encoding for resistance to that drug. The agreement between

these descriptions was evaluated using the Kappa statistic (κ) and unconditional associations (odds ratios (OR)) (18). Each resistance phenotype was evaluated to determine whether it was associated with a summary description of the resistance genes (any resistance genes for that drug; yes or no) and then whether it was associated with each individual resistance gene for that drug.

Three additional sets of analyses were conducted. The first set evaluated the unconditional associations between individual antimicrobial resistance genes. The second set described the unconditional associations between the various resistance phenotypes. The final set of analyses described the unconditional association between each antimicrobial resistance gene and the presence of each virulence factor. All models had a logit-link function, binomial distribution, and exchangeable correlation structure. The association between each variable of interest and outcome was considered significant if $P < 0.05$ and was reported as an odds ratio (OR = expβ) with 95% CI (18). Genes observed in less than 2% of the isolates were not considered in any analyses to avoid problems with model power, stability and convergence. Associations of interest with a zero in the contingency table were noted, and evaluated for significance with a two-sided Fisher's Exact Test (significant if $P < 0.05$).

6.3 Results

Most isolates examined in this study were resistant to at least three of the seven drugs and carried at least three antimicrobial resistance genes (Table 6.3). Only 11% of the isolates were susceptible to all drugs and 9% had no resistance genes. The number of

AMR genes detected in an isolate was a significant predictor of the number of drugs in its resistance phenotype (RR; 1.19, 95% CI; 1.15 to 1.24, $P = 0.0001$).

In this sample, the most common resistance genes identified were *tetA* and *tetB*. The second most common resistance genes were for streptomycin; all isolates with *strA* also carried *strB,* and vice versa. Therefore, all analyses considered these genes as one unit, the *strA–strB* gene pair. Five resistance genes were detected in less than 2% of the isolates (Table 6.4) while 11 were not identified in any of the *E. coli*: bla_{SHV}*, aac(3')-IIa, ant(2")-1a, catI, floR, cmlA, dhfr1b, dhfrV, dhfrVII, dhfrIX* and *dhfrXII.*

On average, 7.6 isolates were tested from each herd (range, 4 to 10). For the three drugs to which phenotypic resistance was most common, more than one type of resistance gene was identified in most herds. All three tetracycline efflux genes were detected in two herds, two were identified in sixteen herds and only one was noted in two herds. All three dihydropteroate synthetase (*sul*) genes were detected in five herds, two in ten herds and one in four herds. One herd had no *sul* genes detected despite having four sulfamethoxazole resistant isolates. One herd had three streptomycin resistance determinants, twelve herds had two, and seven herds had one.

The agreement between resistance, as described by phenotype and genotype, ranged from a kappa of 0.85 for ampicillin to 0.33 for sulfamethoxazole. No outcome had perfect agreement because every antimicrobial-outcome had some isolates with a resistant phenotype but no genetic explanation (Table 6.4). This was most pronounced for

chloramphenicol resistance; no isolates carried any of the three chloramphenicol resistance genes examined. The reverse situation also occurred. Twelve different resistance genes were identified in at least one susceptible isolate. Despite these inconsistencies, each set of resistance genes was a significant predictor of its resistance phenotype, except for resistance to chloramphenicol. When each gene was considered individually, only *sul3* was not a significant predictor of its own phenotypic resistance.

Three genes, *aph(3')-1a*, *dhfrXIII* and *sul3,* were not associated with any other resistance gene. In contrast, *sul1* was associated with five other genes. Three associations showed an increased probability of detecting *sul1* in the presence of another gene, while two suggested a decreased probability (Table 6.5). The strongest association identified was between *aadA1* and *sul1*.

Phenotypic resistance to each drug was associated with phenotypic resistance to at least one other drug, and every identified association was positive (showed an increased probability of observing the outcome resistance in the presence of the predictor resistance) (Table 6.6). The odds ratios could not be estimated for five pairs because no isolates demonstrated one of the combinations of resistance and susceptibility (i.e. 0-0, 0-1, 1-0, or 1-1). However, these resistance combinations were all highly significant on non-parametric analysis (Fisher's Exact; $P < 0.0001$). Importantly, with the exception of the association between *tetA* and *dhfrI*, every significant association between AMR genes (Table 6.5) matched a significant association between the phenotypes encoding for those genes (Table 6.6).

One virulence factor, LT (encoded by *elt*), was not identified and another, VTI (*stx1*), occurred in one isolate. The remaining factors were identified more frequently; intimin (*eae*), 17 isolates; F18 (*fed1)*, 17; STb (*estB*), 23; AIDA (*aida-1*), 49; STa (*estA*), 52; and VT2 (*stx2* or *stx2e*), 58. Thirty-two percent of the *E. coli* isolates did not have any virulence factors identified, while almost half carried two or more (Table 6.1).

The number of antimicrobial resistance genes identified in an isolate was not associated with the number of virulence genes detected *(P* = 0.97), nor was there an association between the presence of any resistance gene and any virulence gene *(P* = 0.92). Three unconditional associations were identified between the presence of a specific antimicrobial resistance gene and a virulence factor. The odds of identifying STb were 4.7 times higher (95% CI; 1.1 to 21, *P* = 0.04) in isolates with *dhfrXIII*. The odds of identifying VT2 were 5.2 times higher (95% CI; 1.7 to 16, *P* = 0.004) in isolates with *dhfrI*. The odds of identifying AIDA were decreased 0.5 times (95% CI; 0.2 to 0.9, *P* = 0.03) in isolates with *tetB*.

6.4 Discussion

This study described the distribution of resistance and virulence genes in *E. coli* from healthy pigs in Alberta and Saskatchewan swine herds. It also described the associations between resistance genes, between resistance phenotypes and between resistance and virulence genes. The frequency of genes and phenotypes in these isolates do not represent the prevalence of these characteristics in the study herds, or other herds in western Canada, because isolate selection was not random. Therefore, prevalence data

should not be extrapolated beyond this study. Despite this limitation, comparing the genes found in these isolates to other North American reports is worthwhile because, to the author's knowledge, other descriptions of AMR genes in *E. coli* from healthy pigs in western Canada are not available

Tetracycline was the most common phenotypic resistance. Although at least 36 genes encode for tetracycline resistance, two efflux genes explained more than 90% of the resistance in these isolates (19). This was similar to other reports of commensal and pathogenic *E. coli* from pigs (2,8,20,21). The next most commonly identified genes encoded for streptomycin resistance. In contrast to an Ontario study where *E. coli* from healthy pigs carried *aadA* genes almost twice as frequently as *strA* - *strB,* these isolates more commonly carried *strA – strB* (8). This finding could reflect regional differences but could also be an artifact of isolate selection. Resistant isolates (MIC $\geq$ 64) were more likely to be selected. These isolates would be more likely to carry *strA* - *strB* because they encode for high level resistance (MIC $\geq$ 64) than the adenylating genes (*aadA*) which encode for MICs < 64 (8,22). The *sul3* gene was first described in 2003 in Switzerland (23). It has since been reported in *E. coli* from pigs in Ontario, Oklahoma and numerous European countries (1,8,24-26). The *sul3* gene appears to be widespread in western Canada as it was the most common sulfonamide resistance gene in this study and was identified in two-thirds of study herds. Finally, resistance to ampicillin was well described by bla_{TEM} which was similar to a description of *E. coli* O149:K91 from sick pigs in Quebec (2).

In contrast to the above resistance types, which were well described by the genes examined, resistance to kanamycin, trimethoprim-sulfamethoxazole and chloramphenicol were poorly explained. Only 59% of the kanamycin and 42% of the trimethoprim-sulfamethoxazole resistant *E. coli* carried a putative resistance gene. Future studies may need to consider more of the known DHFR and kanamycin resistance genes (27,28). Even more notable was the failure to identify any chloramphenicol resistance genes. Although many different chloramphenicol resistance genes have been described, three have been reported in *E. coli* from North America pigs (1,7,29). Resistant *E. coli* from healthy pigs in Ontario and sick pigs in the United States predominately carried *cmlA*, while *catI* and *floR* occurred less frequently (1,7). Considering that chloramphenicol has been banned in Canadian livestock since 1985, resistance has presumably been maintained through co-selection of chloramphenicol genes with other resistance and virulence genes (7,30,31). Future research should investigate what chloramphenicol resistance genes are prevalent in western Canada. Identifying *sul3* in various populations gave insight into how resistance can spread (1,8,23-26). Similarly, describing differences in the chloramphenicol resistance genes between pig populations might provide insight into barriers for the spread or persistence of resistance genes.

Two methods described antimicrobial resistance: measurement of MIC and assessment of resistance genes. These tests had fair (κ 0.2 to 0.4) to almost perfect ($\kappa >$ 0.8) agreement (18). Yet no resistance-outcome was in complete agreement. Phenotypic-resistant isolates with no AMR genes were identified, as were phenotypic-susceptible isolates carrying resistance genes. The apparent contradiction of susceptible isolates

carrying resistance genes has two possible explanations. First, there is a biological explanation. Resistance genes may be unexpressed if they are distant from or associated with a weak promoter in an integron. Similarly, free gene cassettes (not incorporated into an integron) are silent because the integron's promoter is required for expression (32,33). Both of these situations could create a susceptible isolate with resistance genes. Low MIC test sensitivity provides an alternative explanation. Isolates could be falsely categorized as susceptible if the MIC breakpoint is higher than the resistance imparted by the gene. Such a situation is well known with *aadA* genes and streptomycin resistance (8,22).

We previously described the clustering of phenotypic resistance within these herds. Based on those findings, we suggested investigating herd-level risk factors for resistance could be rewarding (Chapter 5). Finding that most herds had more than one type of resistance gene for tetracycline, sulfamethoxazole and streptomycin prompts us to modify that conclusion. Ideally, on-farm studies should explore risk factors for resistance genes rather than phenotypes. Resistance genes encoding for the same drug have different associations with other resistance genes, and so a given antimicrobial exposure would affect these genes differently. For example, the odds of phenotypic trimethoprim-sulfamethoxazole resistance were 4.6 times higher in *E. coli* from herds with sulfonamide use in nursery pigs (Chapter 7). Considering that *dhfrI* was associated more strongly with *sul1* than *sul2*, and *dhfrXIII* was not associated with *sul1* or *sul2*, sulfonamide exposure might select for certain DHFRs but not others. Exploring the relationship between antimicrobial exposure and AMR genes may even explain some of the negative

associations between antimicrobial use and phenotypic antimicrobial resistance found in this thesis and other studies (34) (Chapter Seven).

Positive statistical associations between resistance genes may reflect gene linkages, and thus co-transmission via plasmids, transposons or integrons, while negative odds ratios might indicate gene incompatibilities (7,8,35). For example, the strong association between *aadA-1* and *sul1* might be explained by the presence of *aadA* in gene cassettes and *sul1* in type I integrons, which collect gene cassettes. Plasmid incompatibilities have been proposed as an explanation for negative associations between *tetA* and *tetB* (8). In general, our findings concurred with previous reports (8,25). As in *E. coli* from pigs in Ontario, two sets of resistance genes were observed (8). One set included *tetA*, *aadA-1*, and *sul1* while the other included *tetB*, bla_{TEM}, *strA - strB* and *sul2*. Boerlin et al. did not consider trimethoprim or beta-lactam resistance genes. However, in *E. coli* from various types of meat in Norway, a positive association was noted between *sul1* and *dhfrI* (25). To our knowledge, the association between *tetB* and bla_{TEM} has not been previously reported. Future studies should investigate this gene pair association. Both extended-spectrum beta-lactams and tetracyclines are used in pigs (Chapter 3). If exposure to one drug class increases resistance to the other, barns that use either class, or that cycle between them, could be selecting for resistance to both.

Interestingly, our findings were distinctly different from the associations described in *E. coli* in from sick pigs in Quebec. For example, the association between *sul1* and *tetA* was negative in Quebec but positive in this, and other, studies (2,8,25).

Different *E. coli* types (i.e. ETEC, pathogenic but non-ETEC and commensal) carry different AMR genes (7,8). So, it is plausible that the contradictions between this study and Maynard et al.'s study are because confounding by strain was not accounted for (2).

Multiple drug resistances are generally described as phenotype patterns rather than pair-wise associations between drug-resistances (36,37). While patterns depict the relationships between many drugs, they are difficult to compare between populations. The observed associations between resistance phenotypes were expected because many had been previously reported and all had had been identified in the full *E. coli* collection from which these isolates were sourced (38). However, not all associations from the full set were significant here, reflecting the relatively low power in this subset analysis.

Our most intriguing finding was that almost every associated pair of resistance genes matched an association between their purported phenotypes. The only exception was the association between *tetA* and *dhfrI,* which lacked a corresponding association between tetracycline and trimethoprim-sulfamethoxazole resistance. This suggests associations between resistance phenotypes might predict co-selection. Although this hypothesis requires validation, if proven it would advance our knowledge of co-selection dynamics and provide a cost-effective way to evaluate existing data. Thus, future research should investigate the similarity of gene and phenotype associations in other populations and datasets. Such research would have practical application if it allowed antimicrobial prescribers to consider the effects of specific drug use on other resistance to other drugs.

Similar to antimicrobial resistance genes, *E. coli* virulence-factor genes are often located on plasmids or transposons (7,8). If resistance and virulence genes were linked, any competitive advantage of virulent isolates could explain AMR gene persistence in the absence of antimicrobial selective pressure, and the higher AMR observed in clinical isolates compared to those from healthy animals. Similarly, antimicrobial use could co-select for virulent isolates. In this study, only three virulence and resistance genes were associated and one of those was a negative association. *Escherichia coli* can carry linked resistance and virulence genes (7,8); a hybridization study confirmed some of the numerous statistical associations described between these types of genes (7). However, in those studies, virulence genes predominantly occurred in ETEC but confounding by the three *E. coli* types (ETEC, pathogenic but non ETEC and commensal) was not described (7,8). Hence, our findings could differ from the two previous Ontario reports because of a connection between AMR genes and pathotype. This is plausible considering the *E. coli* virulence genes were associated with specific serotypes, serotypes with pathotypes, and the AMR genes differed between the three *E. coli* types considered (7,8). So although AMU could affect virulence in pathogens, showing that antimicrobial resistance genes and virulence genes are rarely associated in *E. coli* from healthy animals suggests on-farm AMU is not selecting for increased virulence factors in the commensal *E. coli* population.

This study considered the seven resistance phenotypes observed in more than 5% of the *E. coli* from the full dataset. Selection ensured these resistance phenotypes

occurred in more than 10% of these isolates. This restriction minimized problems with statistical power. Due to practical constraints, only some of the resistance genes known for each antimicrobial were considered. Future investigations in this region should consider additional genes for chloramphenicol, trimethoprim and kanamycin resistance as these were poorly explained.

In reality, AMR genes do not interact as isolated pairs but as an interconnected system. While we do not have sufficient data to present these relationships as a causal pathway or multivariate models, associations between resistance genes and phenotypes appear to provide insight into co-selection. If validated, this simple statistical approach may identify unforeseen repercussions from antimicrobial use. This is an important finding, as it would allow policymakers and antimicrobial users to consider co-selection in antimicrobial use decisions.

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Table 6.1 Antimicrobial resistance gene primers, amplicon size and source for 28 genes investigated.

Antimicrobial family	Genetic marker	PCR primer sequence (5' → 3')	Amplicon size (bp)	GenBank accession no.	Source of DNA
Beta-lactams	bla_{TEM} - F	GAGTATTCAACATTTTCGT	857	AF309824	R. C. Levesque
	bla_{TEM} - R	ACCAATGCTTAATCAGTGA			
	bla_{SHV} - F	TCGCCTGTGTATTATCTCCC	768	AF148850	R. C. Levesque
	bla_{SHV} -R	CGCAGATAAATCACCACAATG			
Aminoglycosides	*ant(2")-Ia* (*aadB*)[a] - F	TCCAGAACCTTGACCGAAC	198	AJ238349	Pasteur Institute
	ant(2")-Ia (*aadB*)[a] - R	GCAAGACCTCAACCTTTTCC			
	aac(3)-IIa (*aacC2*) - F	CGGAAGGCAATAACGGAG	591	X75562	R. C. Levesque
	aac(3)-IIa (*aacC2*) - R	TCGAACAGGTAGCACTGAG			
	aac(3)-IV - F	GTGTGCTGCTGGTCCACAGC	705	J05162	R. C. Levesque
	aac(3)-IV - R	AGTTGACCCAGGGCTGTCGC			
	aph(3')-Ia (*aphA1*) - F	ATGGGCTCGCGATAATGTC	701	X92506	A. Huletsky
	aph(3')-Ia (*aphA1*) - R	CTCACCGAGGCAGTTCCAT			
	aph(3')-IIa (*aphA2*) - F	GAACAAGATGGATTGCACGC	700	X04555	R. C. Levesque
	aph(3')-IIa (*aphA2*) - R	GCTCTTCAGCAATATCACGG			
Tetracycline	*tet*(A) -F	GTGAAACCCAACATACCCC	740	X54723	D. Sandvang
	tet(A) - R	GAAGGCAAGCAGGATGTAG			
	tet(B) - F	CCTTATCATGCCAGTCTTGC	627	X01385	J. Harel
	tet(B) - R	ACTGCCGTTTTTTCGCC			
	tetC - F	ACTTGGAGCCACTATCGAC	600	M18329	J. Harel
	*tet*C - R	CTACAATCCATGCCAACCC			
Phenicols	*catI - F*	AGTTGCTCAATGTACCTATAACC	680	V00618	J. Harel
	catI - R	TTGTAATTCATTAAGCATTCTGCC			

Antimicrobial family	Genetic marker	PCR primer sequence (5' → 3')	Amplicon size (bp)	GenBank accession no.	Source of DNA
Phenicols	*floR - F*	CGCCGTCATTCCTCACCTTC	888	X00006	J. Harel
	floR - R	GATCACGGGCCACGCTGTGTC			
	cmlA - F	TTGCAACAGTACGTGACAT	293	AB212941.1	P. Boerlin
	cmlA - R	ACACAACGTGTACAACCAG			
Trimethoprim	*dhfrI - F*	AAGAATGGAGTTATCGGGAATG	774	J01830	J. Harel
	dhfrI - R	GGGTAAAAACTGGCCTAAAATTG			
	dhfrV - F	CTGCAAAAGCGAAAAACGG	881	J01749	J. Harel
	dhfrV - R	AGCAATAGTTAATGTTTGAGCTAAAG			
	dhfrVII - F	GGTAATGGCCCTGATATCCC	827	X65876	S. B. Levy
	dhfrVII - R	TGTAGATTTGACCGCCACC			
	dhfrIX - F	TCTAAACATGATTGTCGCTGTC	853	L06940	M. C. Roberts
	dhfrIX - R	TTGTTTTCAGTAATGGTCGGG			
	dhfrXIII - F	CAGGTGAGCAGAAGATTTTT	823	AF070999	M. C. Roberts
	dhfrXIII - R	CCTCAAAGGTTTGATGTACC			
Sulfonamides	*sul1 - F*	TTCGGCATTCTGAATCTCAC	547	M62822	J. Harel
	sul1 - R	ATGATCTAACCCTCGGTCTC			
	sul2 - F	CGGCATCGTCAACATAACC	543	X53796	Pasteur Institute
	sul2 - R	GTGTGCGGATGAAGTCAG			
	sul3 - F	GAGCAAGATTTTTGGAATCG	880	AY494779.1	P. Boerlin
	sul3 - R	CATCTGCAGCTAACCTAGGGCTTTGGA			
Streptomycin	*strA - F*	CCTGGTGATAACGGCAATTC	546	EF090911.1	P. Boerlin
	strA - R	CCAATCGCAGATAGAAGGC			
	strB – F	ATCGTCAAGGGATTGAAACC	509	EF090911.1	P. Boerlin
	strB - R	GGATCGTAGAACATATTGGC			

Table 6.2 Gene primer sequence, amplicon size and source for eight virulence factors tested in *E. coli.*

Primer name	Primer sequence (5'→3')	Length (base pairs)	*E. coli* positive control	Reference
eae-F	atc ttc tgc gta ctg cgt tca	790	STJ348	Beudry
eae-R	cat tat gga acg gca gag gt		O157:H7	
stA-F	tcc cct ctt tta gtc agt caa ctg	171	P97-2554B	So, Nglekea
stA-R	gca cag gca gga tta caa caa agt		O149:K91	
stB-F	gca ata agg ttg agg tga t	377	P97-2554B	Lortie
stB-R	gcc tgc agt gag aaa tgg ac		O149:K91	
VT1-F	tta gac ttc tcg act gca aag	530	STJ348	Woodward
VT1-R	tgt tgt acg aaa tcc cct ctg		O157:H7	
VT2-F	cta tat ctg cgc cgg gtc tg	327	STJ348	Woodward
VT2-R	aga cga aga tgg tca aaa cg		O157:H7	
fedA1-F	gtg aaa aga cta gtg ttt att tc	510	P88-1199	Imberechts
fedA1-R	ctt gta agt aac cgc gta agc		O139:K82	
Aida1-F	aca gta tca tat gga gcc a	585	2787	Benz, Ngeleka
Aida1-R	tgt gcg cca gaa cta tta		2787	
LT-F	tta cgg cgt tac tat cct ctc ta	275	P97-2554B	Furrer
LT-R	ggt ctc ggt cag ata tgt gat tc		O149:K91	

Table 6.3 Number of drug resistances, antimicrobial resistance genes and virulence genes in commensal *E. coli* (n = 151).

Number of drug-resistances or genes	Number of isolates		
	Drug phenotypes	Resistance genes	Virulence genes
Zero	16	13	49
One	3	24	28
Two	24	29	45
Three	37	17	22
Four	35	34	3
Five	25	21	2
Six	11	10	1
Seven	0	1	1
Eight	0	2	0

Table 6.4 Resistant *E. coli* (P+) with no resistance gene (G-), and susceptible isolates (P-) with resistance genes (G+); associations between drug-phenotypes and resistance genes and phenotype:genotype inter-test agreement (n=151).

Antimicrobial	N_P	Genes	N_G	P+ / G-	P- / G+	Odds ratio	95% confidence interval	*P*	Kappa
Ampicillin	69	any	60	10	1	534	54 - 5280	<0.0001	0.85
		bla$_{TEM}$	60		1	534	54 - 5280	<0.0001	
Chloramphenicol	48	any	0	48	0				
Kanamycin	34	any	21	14	1	55	16 – 182	<0.0001	0.67
		aph(3')-1a	16		0	32	9.4 - 108	<0.0001	
		aph(3')-IIa	3		0			-	
		aac(3)-IV	*2*		*1*			-	
Streptomycin	82	any	79	20	17	10	5.3 - 20	<0.0001	0.51
		aadA1	35		10	2.5	1.2 - 5.3	0.01	
		aadA6	1		0			-	
		strA - strB	51		7	11	5.3 - 22	<0.0001	
Sulfamethoxazole	111	any	69	42	8	3.3	2.6 - 16	<0.0001	0.33
		sul1	22		1	9.7	1.2 - 78	0.03	
		sul2	27		1	13	1.2 - 142	0.03	
		sul3	40		6	2.7	0.9 - 8.0	0.06	
Tetracycline	125	any	124	5	4	95	29 - 312	<0.0001	0.77
		tetA	52		3	3.5	1.2 - 10	0.03	
		tetB	81		1	17	4.6 - 61	<0.0001	

Antimicrobial	N	Genes	N	P+ / G-	P- / G+	Odds ratio	95% confidence interval	*P*	Kappa
Tetracycline		*tetC*	2		1	0.4	0.1 - 3.4	-	
Trimethoprim-sulfamethoxazole	24	any	14	14	4	23	4.6 - 116	<0.0001	0.46
		dhfrI	7		3	8.3	1.6 - 44	0.01	
		dhfrXIII	6		1	35	2.0 - 619	0.01	
		dhfrXV	1		0			-	

N_P: Number of *E. coli* expressing phenotypic resistance to antimicrobial
N_G: Number of *E. coli* carrying resistance gene
P+ / G-: Number of phenotypically resistant *E. coli* with no resistance gene for that drug identified
P- / G+: Number of phenotypically susceptible *E. coli* with a resistance gene for that drug identified

Table 6.5 Significant odds ratios between *E. coli* resistance genes and 95% confidence intervals.

Outcome	Predictor	Odds Ratio	95% confidence interval	*P*
bla_{TEM}	*strA - strB*	2.4	1.0 - 5.4	0.04
	tetB	2.9	1.4 - 5.8	0.003
aadA1	*sulI*	70.4	12.0 - 412.0	0.0001
	tetA	7.8	2.7 - 23.0	0.0002
strA - strB	bla_{TEM}	2.4	1.0 - 5.5	0.04
	sul1	0.2	0.1 - 0.7	0.01
	sul2	6.8	2.9 - 16.3	0.0001
	tetA	0.3	0.2 - 0.7	0.003
	tetB	4.3	1.9 - 9.8	0.0004
sul1	*aadA1*	76.8	14.7 - 402.2	0.0001
	strA - strB	0.2	0.0 - 0.6	0.005
	tetA	9.0	3.2 - 25.2	0.0001
	tetB	0.4	0.2 - 0.9	0.04
	dhfrI	8.4	1.6 - 43.6	0.01
sul2	*strA - strB*	8.5	3.4 - 21.7	0.0001
tetA	*aadA1*	8.1	2.7 - 24.4	0.0002
	strA - strB	0.3	0.1 - 0.6	0.001
	sul1	8.6	3.0 - 24.1	0.0001
	tetB	0.1	0.0 - 0.1	0.0001
	dhfrI	4.7	1.2 - 18.0	0.02
tetB	bla_{TEM}	2.9	1.5 - 5.8	0.002
	strA - strB	4.3	1.9 - 9.8	0.0005
	tetA	0.1	0.1 - 0.2	0.0001
dhfrI	*sul1*	9.4	1.8 - 48.0	0.007
	sul2	4.5	1.4 - 14.1	0.01

Table 6.6 Significant odds ratios between *E. coli* drug-resistance phenotypes and 95% confidence intervals.

Outcome	Predictor	Odds Ratio	95% confidence interval	*P*
Ampicillin	Streptomycin	2.8	1.6 - 4.7	0.0002
	Tetracycline	11.0	3.4 - 35.3	0.0001
	Trimethoprim-Sulfamethoxazole	3.5	1.1 - 10.4	0.03
Kanamycin	Streptomycin	2.6	1.3 - 5.0	0.01
Streptomycin	Ampicillin	3.2	1.8 - 5.8	0.0001
	Kanamycin	3.0	1.1 - 8.4	0.04
	Tetracycline	**		<.0001
	Sulfamethoxazole	2.0	1.2 - 3.4	0.01
Tetracycline	Ampicillin	16.6	3.9 - 70.7	0.0001
	Streptomycin	**		<.0001
	Sulfamethoxazole	7.8	3.9 - 15.7	0.0001
Sulfamethoxazole	Streptomycin	1.9	1.1 - 3.3	0.02
	Tetracycline	8.4	4.3 - 16.4	0.0001
	Trimethoprim-Sulfamethoxazole	**		<.0001
	Chloramphenicol	**		<.0001
Trimethoprim-Sulfamethoxazole	Ampicillin	3.5	1.2 - 10.1	0.02
	Sulfamethoxazole	**		<.0001
Chloramphenicol	Sulfamethoxazole	**		<.0001

CHAPTER 7
ASSOCIATIONS BETWEEN FEED AND WATER ANTIMICROBIAL USE IN FARROW-TO-FINISH SWINE HERDS AND ANTIMICROBIAL RESISTANCE OF FECAL *ESCHERICHIA COLI* FROM GROW-FINISH PIGS

7.1 Introduction

Swine veterinarians and producers use antimicrobials to treat and prevent bacterial infections, improving pig health and welfare. Antimicrobials are also used as feed additives to increase daily gain and improve feed efficiency (1-3). Although antimicrobials are indispensable tools for managing bacterial disease, their use also contributes to acquired antimicrobial resistance (AMR) in commensal and pathogenic bacteria (1,4-10).

Resistant bacteria can survive and propagate in the presence of an antimicrobial. Some bacteria are intrinsically resistant, while others develop resistance through chromosomal mutations or acquiring resistance genes (5,11). Horizontal transmission of resistance genes occurs through plasmids, transposons, and integrons. These elements can carry multiple resistance genes and transmit them as a unit (5,12-14). Therefore, bacteria with acquired AMR are frequently resistant to unrelated drugs.

Resistant zoonotic pathogens, such as *Salmonella* spp., pose a direct risk to consumers eating contaminated pork (15,16). Resistant pathogens have an higher infection rate, limited treatment options and increased virulence (4,17,18). Resistant commensal organisms are also a food safety hazard. Although the magnitude of this risk is undefined, resistance genes may spread to other bacteria in the human gastrointestinal tract (6,10,19-21). Furthermore,

commensal bacteria reflect the selective pressures for resistance to develop in the normal intestinal flora (6,22). Research projects and surveillance programs often use generic *Escherichia coli* as a model for AMR in gram-negative commensals (8,9,22-25). The ubiquity of *E. coli* allows AMR to be compared between populations of healthy animals (6,22).

Experimentally, antimicrobial exposure increases the prevalence of resistant *E. coli* in healthy pigs (7,8,26). On-farm studies have also shown antimicrobial use is associated with *E. coli* AMR (9,27,28). Observational studies are ideal for investigating complex problems with many causes or hypotheses (29). These studies can consider numerous drug exposures and various resistances simultaneously. To date, observational investigations have not considered the extent of antimicrobial exposure within herds (9,27). Therefore, the objective of this project was to investigate the dose response relationships between on-farm antimicrobial use and AMR in *E. coli* from grow-finish pigs.

7.2 Materials and Methods

7.2.1 Herd enrollment, sample collection and laboratory methods

A convenience sample of 20 herds was allocated to eight swine veterinarians in Saskatchewan (thirteen herds) and Alberta (seven herds). Herds were selected by the veterinarians and met the inclusion criteria of having more than 100 sows and participation in the Canadian Quality Assurance® (CQA®) Program (30). Each veterinarian enrolled two to four herds. Study herds were visited once between May and September of 2004. Antimicrobial use data and information on inventory and production practices were collected during herds visits (Table 7.1). As previously described, pen-level fecal samples collected from healthy grow-finish

pigs were cultured for *E. coli*. Harvested isolates were tested for susceptibility to sixteen antimicrobials (Chapter 5).

7.2.2 Antimicrobial use data collection – Exposure through feed and water

Data collection of antimicrobial use through feed and water was described in detail (Chapter 3). Briefly, each herd owner or manager used existing records to complete surveys on antimicrobial use through feed and water (Appendix A). Data for each exposure of suckling, nursery, grow-finish pig, or sows in the previous 12 months included the product used, number of pigs exposed, and duration of exposure. Antimicrobial use was described as the incidence per pig-day and scaled by 1,000 or 100,000 to facilitate interpretation (Equation 1). Pigs could be exposed more than once per day, either through products in both feed and water, or through products containing multiple antimicrobials.

Equation 1. Formula for Antimicrobial Exposure Incidence (AEI)

$$AEI = [Pigs_E * Days_E] / [Pigs_R * Days_R]$$

E = exposed

R = at risk

7.2.3 Statistical analysis

Antimicrobial exposure and susceptibility data were maintained in a relational database (Microsoft Access, Microsoft Corporation, Redmond, Washington, USA). Descriptive statistics were calculated using commercially available software (Microsoft Excel, Microsoft Corporation, Redmond, Washington, USA). Intermediate MIC values were classified as susceptible for all analyses. Statistical models, adjusted for herd-level clustering using generalized estimating

equations (GEE) (PROC GENMOD, SAS version 9.1, SAS Institute Inc., Cary, North Carolina), had a logit-link function, binomial distribution, and an exchangeable correlation structure. When necessary, extra-binomial variation was allowed by a scale parameter equal to the square root of the Pearson's Chi-Square divided by the degrees of freedom in the model.

The prevalence of resistance to each antimicrobial was calculated using the intercept (β_0) and 95% confidence intervals (CI) from a null binomial response model in $1/ [1 + \exp(-\beta_0)]$ (29). A risk factor model was developed for each resistance that was observed in more than 5% of the isolates. Models were restricted to these drug resistances to avoid problems with power, model stability and convergence associated with infrequent outcomes.

In contrast to AMR, which was described for individual drugs, antimicrobial exposures were described by class: The number of farms using each antimicrobial was insufficient to evaluate the dose response relationship between individual drugs and resistance while sufficient between-farm variability occurred when antimicrobial were grouped by class. An antimicrobial class refers to drugs with similar chemical structures and mechanisms of action (31,32). The antimicrobials used by study herds were classified as follows: i) aminoglycosides (included aminocyclitols) ii) beta-lactams iii) macrolides (included lincosamides and pleuromutilins) iv) sulfonamides and v) tetracyclines. Antimicrobial use was further stratified by the production phase exposed: suckling, nursery, grow-finish pigs and sows. Antimicrobial use variables used in five or more herds were modeled as the exposure incidence (continuous). Antimicrobial use variables used in less than five herds were collapsed to 'any use' versus 'no use' (dichotomous). Those used by only one herd were not evaluated.

The outcome of each model was the proportion of isolates from a sample that were resistant to the antimicrobial. Each antimicrobial use variable was screened to determine its unconditional association with the outcome variable; only those significant at $P < 0.2$ were considered further. For continuous antimicrobial use variables, the effect estimate of each quartile was graphed against the log odds of the outcome to assess the relationship for linearity. Non-linear associations were addressed by categorizing antimicrobial use as no use, low use (0.1 to 500 exposures per 1000 pig-days), and high use (>500 exposures per 1000 pig-days) because this categorization best fit the variable distribution. Categorical variables were considered further if significant at $P < 0.2$.

All antimicrobial use variables with an unconditional $P < 0.2$ were included in the full model. Manual step-wise backward selection was used to develop a main effects model, retaining only antimicrobial use variables significant at $P < 0.05$. Variables describing exposure to the same antimicrobial class as the outcome were retained, regardless of significance, until all other non-significant variables were eliminated. For example, when modeling resistance to sulfamethoxazole, variables describing sulfonamide use were retained until all non-significant variables describing other classes were removed. This modeling decision was based on the premise that direct selection and cross-resistance affect resistance rates more strongly than co-selection.

Variables removed from the full model were re-introduced into the main effects model separately to ensure they had not been inappropriately removed because of confounding. Two-

way interactions were considered between exposures in the same swine production phase. Interaction terms, significant at $P < 0.05$, were retained in the final model along with their main effects. Production variables were considered as potential confounders (Table 7.1). Those that altered a parameter estimate by more than 25% were retained in the final model. Residuals of the final models were examined visually for outliers. The association between each variable of interest and the outcome was reported as an odds ratio (OR = expβ) with 95% CI (29).

7.3 Results

Four hundred and five samples were cultured for *E. coli*. On average, 63 isolates were harvested per herd (range, 60 to 88) for a total of 1439 isolates. More than 5% of the *E. coli* were resistant to seven of the sixteen antimicrobials in the panel (Table 7.2). Resistance to ampicillin, streptomycin, sulfamethoxazole and tetracycline were each observed in isolates from every herd. One herd had no isolates resistant to chloramphenicol, four had no isolates resistant to trimethoprim-sulfamethoxazole, and five had no isolates resistant to kanamycin.

In every herd, antimicrobials had been used in feed or water in the previous 12 months. Nursery pigs were exposed to antimicrobials most frequently while sows were exposed least frequently (Table 7.3). Macrolides were used in 18 of 20 herds, tetracyclines in 15, aminoglycosides in 9, beta-lactams in 9, and sulfonamides in 7 (Table 7.4).

Seven *E. coli* resistance outcomes were modeled against antimicrobial exposures through feed and water (Table 7.2). Of these seven antimicrobials, tetracycline was the only antimicrobial that was both considered as a resistance outcome and was used in feed or water in study herds. Other tetracycline exposures included oxytetracycline and chlortetracycline. Resistance to

sulfamethoxazole was also modeled; because the sulfonamide derivative was not always reported we cannot definitively state this drug was not used in study herds. However, sulfamethoxazole is not licensed for use in Canadian pigs while the following sulfonamides are licensed for administration through feed or water: sulfadoxine, sulfaguanidine, sulfamerazine, sulfamethazine, sulfanilamide, sulfapyridine and sulfathiazole (33). Sulfonamide exposures were likely a mixture of these. Resistance to two aminoglycosides, kanamycin and streptomycin, were modeled. Neither of these drugs were used in study herds but another aminoglycoside (neomycin) and an aminocyclitol (spectinomycin) were used. Two of the antimicrobials to which resistance was modeled were administered parenterally but not in feed or water. Trimethoprim, in combination with sulfadoxine, was used as an injectable product and thus was not captured by any of the antimicrobial exposure variables. Similarly, ampicillin was also only used as an injectable drug. Beta-lactam use was predominantly penicillin G, although nursery pigs received amoxicillin through water in one herd. Resistance to chloramphenicol was the only outcome where no product from its antimicrobial class was used in study herds. In contrast, macrolides were used extensively but had no resistance outcomes tested. Macrolide exposure included tylosin and tilmicosin, tiamulin, and lincomycin.

Six of the seven antimicrobial resistance outcomes had significant antimicrobial use risk factors (Table 7.5). Five resistance outcomes were associated with exposure to drugs in the same antimicrobial class. Resistance to tetracycline was only associated with exposure to tetracyclines in nursery pigs (Figure 1), while resistance to ampicillin was only associated with beta-lactam use in grow-finish pigs (Table 7.5). In contrast, *E. coli* resistance to kanamycin and streptomycin were not associated with aminoglycoside exposure. Resistance to streptomycin was predicted by

macrolide exposure in grow-finish pigs (Figure 2), (Table 7.5). Resistance to kanamycin was the only outcome with no significant antimicrobial use risk factors.

Resistance to sulfamethoxazole was linearly associated with sulfonamide and aminoglycoside exposure in nursery pigs. Resistance to sulfamethoxazole was also predicted by macrolide use in grow-finish pigs; *E. coli* from herds using a high rate had 3.4 times higher odds (95% CI, 1.9 to 6.1; P <0.0001) of resistance than isolates from herds using a low rate, and 6.4 times (P <0.0001) higher odds of resistance than *E. coli* from herds using none. The odds of resistance to sulfamethoxazole did not differ significantly between isolates from herds using a low rate of macrolides in grow-finish pigs compared to herds with no use (P = 0.07).

The odds of trimethoprim-sulfamethoxazole resistance increased with four antimicrobial use variables, including sulfonamide use in nursery pigs (Table 7.5). In contrast, nursery pig exposure to tetracycline was associated with decreased odds of resistance. Resistance to trimethoprim-sulfamethoxazole was the only outcome associated with antimicrobial use in sows. It was also the only model with an important confounder; *E. coli* from herds selling breeding stock were more likely to be resistant to trimethoprim-sulfamethoxazole than isolates from herds that only sold pigs for consumption.

The odds of *E. coli* resistance to chloramphenicol increased with aminoglycoside exposure and decreased with tetracycline exposure in suckling pigs (Table 7.5). Macrolide use in grow-finish pigs was also significant; the odds of chloramphenicol resistance were 4.1 times higher (95% CI, 2.2 to 7.6; P <0.0001) in isolates from herds with high exposure compared to

low exposure, and 5.9 times higher (P = .0006) in isolates from herds with high exposure compared to no exposure. The odds of resistance to chloramphenicol were not significantly different between *E. coli* from herds with low macrolide exposure compared to those with no exposure (P = 0.4).

7.4 Discussion

Antimicrobial resistance is a natural consequence of drug selective pressures. In swine herds, these pressures may be intensified because of long-term antimicrobial exposure through water and feed (34). Therefore, this study investigated the link between herd level antimicrobial use, through feed and water, and resistance of *E. coli.* Although this study considered *E. coli* from grow-finish pigs only, AMR was associated with antimicrobial use in all production phases. This indicates that antimicrobial use in pigs distant from market may have food safety repercussions. Four of the resistance outcomes were associated with exposure to unrelated drugs, indicating co-selection (11,12). While co-selection is familiar to scientists, producers and veterinarians need to understand this phenomenon before AMR can be addressed at the herd-level.

In general, the statistical models generated by this study indicate that antimicrobial exposure provides a competitive advantage to resistant *E. coli*. Three aspects of acquired AMR guided our interpretation of these models. Direct selection occurs when exposure to a drug results in increased resistance to the same drug. Cross-resistance occurs when resistance to a drug automatically confers resistance to a related drug. Finally, exposure to any drug in a multiple drug resistant phenotype creates selective pressure for every resistance in the phenotype (co-selection) (11,12). The association between tetracycline resistance and exposure was

interpreted as both direct selection and cross selection, while the association between sulfamethoxazole resistance and sulfonamide use implied cross selection, and the association between chloramphenicol resistance and aminoglycoside exposure suggested co-selection.

Enterobacteriaceae are intrinsically resistant to penicillin G (35). As penicillin G was the only beta-lactam used in grow-finish pigs, the association between ampicillin resistance and beta-lactam exposure does not fit our understanding of cross selection. From a mechanistic perspective, low rates of exposure to a drug that is inactive against *E. coli* would be unlikely to cause this increase in ampicillin resistance. Rather, it seems more plausible that beta-lactam exposure was a proxy for a common, undefined risk factor. However, the possibility that penicillin G exposure is a true risk factor for ampicillin resistance cannot be discounted; others have reported an association between ampicillin resistance in *E. coli* from grow-finish pigs and penicillin G use in lactating sows (9). Investigating this association in more herds with a wider range of beta-lactam exposures or in a controlled laboratory experiment might clarify this finding.

Neomycin and spectinomycin were used in study herds, yet aminoglycoside use was not associated with resistance to kanamycin or streptomycin. This was unexpected because resistance genes encoding for both streptomycin and spectinomycin (*strA / strB, aadA*) have been reported in *E. coli* from Canadian pigs (36,37). Thus, assuming spectinomycin directly selects for resistance, it should also select for resistance to streptomycin. Such a relationship between spectinomycin use and streptomycin resistance has been previously reported (27). A similar relationship exists between kanamycin and neomycin; resistance to both drugs is encoded by

aac(3)IV and *aph(3')-Ia* genes, which have been identified in *E. coli* from Canadian pigs (36,37). This study's design might explain the failure to identify associations between aminoglycoside resistance and exposure. Aminoglycosides were only used in suckling and nursery pigs while *E. coli* were from grow-finish pigs. Other researchers have observed that *E. coli* resistance to apramycin declines rapidly following the removal of apramycin from feed. Thus, if aminoglycoside use had an effect on resistance, it may have dissipated before isolate collection in this study (8,38).

Finding chloramphenicol resistance in these *E. coli* demonstrates that antimicrobial resistance can persist without direct selection. Canadian authorities banned chloramphenicol use in food animals in 1985 and no study herds reported its use or use of the related approved drug florfenicol (39). Antimicrobial exposure can increase resistance to unrelated drugs by selecting for multiple resistant organisms (12,14). Others have reported physical linkages and statistical associations between chloramphenicol and aminoglycoside resistance genes. This supports the conclusion that chloramphenicol resistance might be persisting because of aminoglycoside use in suckling piglets and macrolide use in grow-finish pigs (40,41). A report of increased odds of chloramphenicol resistance in herds adding tylosin to finisher diets further supports this statement (27).

Identifying macrolide use as a risk factor for four different resistance outcomes was striking because *E. coli* are intrinsically resistant to macrolides (42,43). However, another report also found macrolide use to be a risk factor for various *E. coli* resistances (27). In humans, when erythromycin is administered orally, the intestinal concentration overcomes the intrinsic

resistance of *E. coli* (42,44). This suppresses the aerobic gram-negative intestinal flora and selects for erythromycin resistance (42,44). *Escherichia coli,* isolated from people receiving erythromycin, can carry transmissible plasmids with linked erythromycin, ampicillin, gentamicin and streptomycin resistance genes (42,44). In pigs, *E. coli* MICs to tylosin, tilmicosin, lincomycin and tiamulin have not been investigated. The intestinal and colonic concentrations of these antimicrobials have only been estimated (45). Considering the situation in humans, it is plausible that macrolide resistant *E. coli* exist in pigs. Investigation into macrolide use and resistance in *E. coli* from pigs is urgently needed considering tylosin is among the most commonly used antimicrobials in grow-finish pigs and use of this antimicrobial is considered critically important in some classification schemes (32,34,46-48).

Similar to other observational studies, antimicrobial use in other production phases was associated with *E. coli* AMR in grow-finish pigs in this study (9,27,49). These findings have been supported experimentally; offspring from sows receiving oxytetracycline in feed pre-farrowing had increased oxytetracycline resistance compared to control piglets (8). Thus, AMR studies should consider antimicrobial use in the entire herd as potential risk factors. Equally important, antimicrobials should always be used sparingly in all ages of pigs.

Two of our statistical models identified decreased odds of resistance with increased antimicrobial exposure. Similar to this study, Akwar reported tetracycline exposure was protective for chloramphenicol resistance (27). Finding decreasing resistance with increased antimicrobial use is counter-intuitive. When statistical models identify counter-intuitive associations, they may be discounted as spurious. In our case, corroborating findings between

studies strengthens the premise that the statistical associations reflect biological reality and underlines the importance of heeding unexpected or conflicting results. As recommended in Chapter 6, the chloramphenicol resistance genes in these isolates require further description. Others have reported negative associations between *catA1* and *tetB* (40). Further investigation in these isolates might also demonstrate incompatible tetracycline and chloramphenicol resistance genes.

The frequency of AMR in these *E. coli* was similar to surveillance reports from abattoirs and swine herds in North America (9,23,24,27). Although descriptions of antimicrobial use in North American pigs are scarce, the number of herds using group medications and the types of drugs used in the current study were comparable to available data (34,47,50). The similarity of the antimicrobial use and resistance between this and other reports, and the infrequent confounding by management variables, suggests that these findings are relevant to many swine farms in North America.

Reporting relationships between AMR and antimicrobial exposure incidence through feed and water is unique. Previous risk factor studies have described antimicrobial use in feed and water qualitatively, while we described the rate of exposure (9,27). Assuming a causal relationship, long term antimicrobial use in pigs affects AMR more than targeted use. Despite this strength, our description of antimicrobial use was also a primary weakness of this study. Describing antimicrobial exposure by class allowed consideration of dose-response relationships but may have biased associations towards the null. Pharmacokinetic characteristics, such as the rate of absorption, and physical characteristics, such as stability in gastric acid, can differ

between drugs within a class (31). If the selective pressure for resistance differed markedly between drugs within a class, this study may have missed significant associations because exposure was defined by antimicrobial class. Future studies will need to enroll more herds or purposively select herds based on antimicrobial use to evaluate individual drugs as risk factors.

Administering antimicrobials to pigs is a risk factor for antimicrobial resistance. However, the human health risks from resistant porcine *E. coli* are unquantified and the risk factors for many resistances are unknown. Until more data are available, the swine industry faces the conundrum of knowing that antimicrobial use can select for resistance without knowing which antimicrobials might pose lower risks to consumers (32,51). Until such knowledge is generated, swine producers should use antimicrobials judiciously to mitigate the potential effects of resistance. These findings emphasize the importance of co-selection and minimizing drug use to decrease selective pressures for multiple drug resistances.

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Table 7.1 Study herd (n = 20), grow-finish production phase (n = 20) and sampled pen (n = 405) characteristics: frequency, or mean and standard deviation (*S*).

Level	Characteristic		Description
Herd			
	Number of sows in herds (median and IQR)[a]		456 (274 - 1042)
	Days in each production phase	Suckling	21 (1.8)
		Nursery	46 (15.7)
		Grow-finish	112 (27.1)
	Purpose for which animals were sold*	Breeding stock	7
		Slaughter only	13
	Number of sites farrow-to-finish herd located on*	one	15
		two	2
		three	3
Grow-Finish Phase			
	All-in-all-out by room or barn*	yes	9
		no	11
	Pens emptied between batches*	yes	12
		no	8
	Manure removed between batches*	yes	10
		no	10
	Pens washed and disinfected between batches*	yes	8
		no	12
	Times barn was washed in previous 12 months*		3 (3.7)
	Days pens empty between batches*		1 (1.7)
Pen			
	Sex [b, *]	Boar	20
		Gilt	167
		Barrow	134

Level	Characteristic		Description
		Mixed	80
	Flooring type*	Full slat	102
		Partial slat	299
		Solid	4
	Feed presentation*	Feeder	362
		Floor	43
	Weeks pigs had been in pen at sampling*		5.9 (4.1)
	Pen density (pigs per m^2)*		1.7 (0.8)

a: non-normally distributed
b: data missing for four pens
* considered as potential confounders

Table 7.2 Population average prevalence of resistance in *E. coli* (n = 1439), 95% confidence intervals (CI), dilution range tested and resistance breakpoint (resistant if ≥ MIC).

Antimicrobial	Prevalence	95% confidence interval	Dilution range	Resistant MIC
Tetracycline*	66.8	57.9 - 74.7	4 - 32	16
Sulfamethoxazole*	46.0	36.4 - 56.0	16 - 512	512
Streptomycin*	33.4	27.8 - 39.5	32 - 64	64
Ampicillin*	18.6	17.9 - 19.2	1 - 32	32
Chloramphenicol*	17.3	10.7 - 26.7	2 - 32	32
Kanamycin*	9.3	4.6 - 18.0	8-64	64
Trimethoprim-Sulfamethoxazole*	7.4	5.1 - 10.5	0.12 / 2.38 - 4 / 76	4 / 76
Cephalothin	3.8	2.2 - 6.6	2 - 32	32
Gentamicin	0.8	0.4 - 1.8	0.25 - 16	16
Cefoxitin	0.6	0.2 - 1.6	0.5 - 16	32
Amoxicillin-Clavulanic Acid	0.4	0.1 - 1.3	1 / .05 - 32 / 16	32 / 16
Ceftiofur	0.1	0.0 - 0.5	0.12 - 8	8
Nalidixic Acid	0.1	0.0 - 0.5	0.5 - 32	32
Amikacin	0.0	0.0 - 0.2	0.5 - 4	64
Ceftriaxone	0.0	0.0 - 0.2	0.25 - 64	64
Ciprofloxacin	0.0	0.0 - 0.2	0.015 - 4	4

* risk factor models developed for resistance phenotypes observed in more than 5% of the isolates

Table 7.3 Antimicrobial exposure incidence per 1000 pig-days for all antimicrobial classes combined. Incidence in all herds (n = 20) and in herds with an exposure incidence greater than zero.

Production	AEI of all herds		AEI in herds with incidence > 0		
Phase	Median	IQR	Herds	Median	IQR
Suckling	3	0 - 750	10	833	552 - 1056
Nursery	929	663 - 1230	20	929	663 - 1230
Grow-finish	301	16 - 965	15	715	160 - 1000
Sow	0	0 - 20	8	49	15 - 199

Table 7.4 Frequency of antimicrobial use by class and production phase, (n = 20 herds) and description of exposure incidence per 1,000 pig-days in herds with an exposure incidence greater than zero.

Antimicrobial class	Production phase	N	Herds reporting AEI > 0		
			Median	IQR[a]	
Aminoglycosides	Suckling	3	700	517	739
	Nursery	8	272	137	501
	Grow-finish	0	-	-	-
	Sow	0	-	-	-
Beta-lactams	Suckling	3	286	238	310
	Nursery	8	210	176	291
	Grow-finish	3	11	6	17
	Sow	1	64	-	-
Macrolides	Suckling	8	310	228	572
	Nursery	17	528	411	974
	Grow-finish	15	714	160	977
	Sow	3	34	18	167
Sulfonamides	Suckling	3	286	238	310
	Nursery	6	143	126	191
	Grow-finish	1	24	-	-
	Sow	0	-		
Tetracyclines	Suckling	5	333	273	381
	Nursery	13	226	220	508
	Grow-finish	1	725	-	-
	Sow	5	37	16	185

a: IQR = Inter-quartile range is presented for herds with exposure incidence > 0

Table 7.5 Final logistic regression models for *E. coli* resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and trimethoprim-sulfamethoxazole (n = 1439 isolates, n = 405 pens).

Resistance outcome	Antimicrobial Use Risk Factor			Odds Ratio	95% confidence interval	*P*
Ampicillin	Grow-finish	Beta-lactam	any use	2.8	1.6 - 5.0	0.0005
			no use	reference		
Chloramphenicol	Suckling	Aminoglycoside	any use	2.3	1.2 - 4.6	0.02
			no use	reference		
	Grow-finish	Macrolide	high	5.9	2.2 - 16.0	0.04
			low	1.4	0.6 - 3.4	
			no use	reference		
	Suckling	Tetracycline	100,000 pig-days	0.8	0.6 - 0.97	0.02
Streptomycin	Grow-finish	Macrolide	100,000 pig-days	1.07	1.01 - 1.1	0.02
Sulfamethoxazole	Nursery	Aminoglycoside	100,000 pig-days	1.1	1.02 - 1.3	0.02
	Grow-finish	Macrolide	high	6.4	3.3 - 12.2	0.004
			low	1.9	1.0 - 3.7	
			no use	reference		
	Nursery	Sulfonamide	100,000 pig-days	2.0	1.4 - 2.9	0.0001

Resistance outcome	Antimicrobial Use Risk Factor			Odds Ratio	95% confidence interval	*P*
Tetracycline	Nursery	Tetracycline	100,000 pig-days	1.3	1.1 - 1.5	0.0004
Trimethoprim-sulfamethoxazole	Nursery	Sulfonamide	any use	4.6	2.0 - 10.5	0.0004
			no use	reference		
	Sow	Macrolide	any use	3.9	1.6 - 9.6	0.003
			no use	reference		
	Nursery	Tetracycline	100,000 pig-days	0.9	0.8 - 1.0	0.047
	Sow	Tetracycline	100,000 pig-days	1.6	1.1 - 2.3	0.008
	Grow-finish	Beta-lactam	any use	7.9	2.8 - 21	<.0001
			no use	reference		
	Herd type	Commercial		2.6	1.5 - 4.9	0.004
		Breeding stock		reference		

Figure 7.1 Relationship between tetracycline use in nursery pigs and tetracycline resistance in *E. coli* from grow-finish pigs

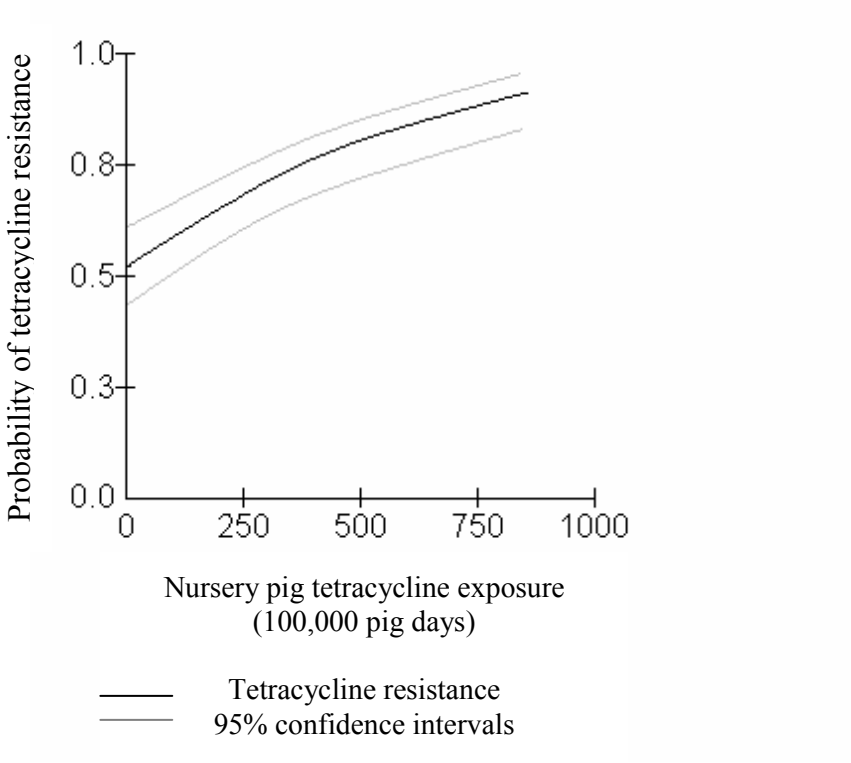
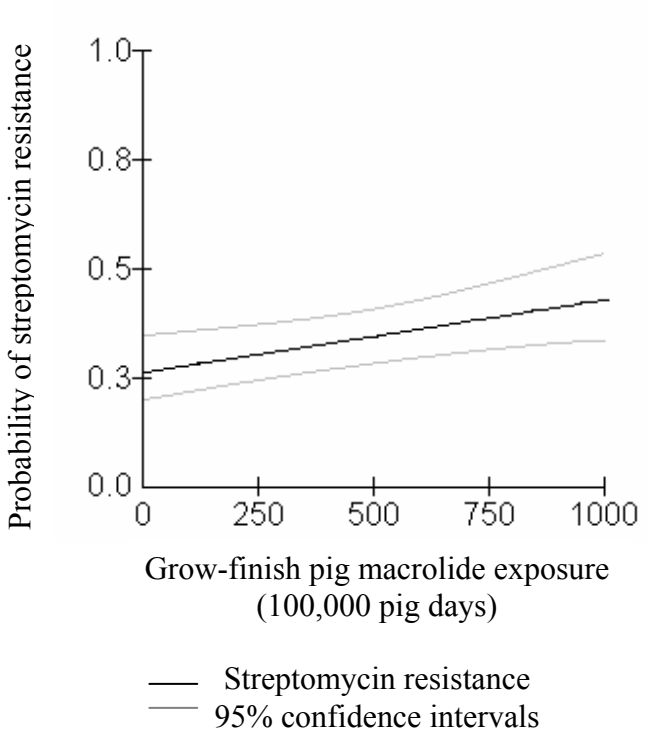


Figure 7.2 Relationship between macrolide use in grow-finish pigs and streptomycin resistance in *E. coli* from grow-finish pigs



CHAPTER 8
ASSOCIATIONS BETWEEN ANTIMICROBIAL EXPOSURE AND RESISTANCE IN FECAL *CAMPYLOBACTER* SPP. FROM GROW-FINISH PIGS ON-FARM IN ALBERTA AND SASKATCHEWAN CANADA

8.1 Introduction

Campylobacter species are a leading cause of bacterial enteritis for people in many counties, including Canada (1,2). *Campylobacter jejuni* are responsible for 80 to 90% of clinical disease, while *Campylobacter coli* cause 5 to 10% of cases (2,3). Although most enteric *Campylobacter* infections are self-limiting, people requiring antimicrobial therapy are often treated with a fluoroquinolone or macrolide. Hence, the growing resistance to these drugs is a serious public health problem (4-7). This problem may be linked to antimicrobial use in food animals because *Campylobacter* infections are primarily foodborne (1,5).

Pigs are an important reservoir for *C. coli*; most swine herds are *Campylobacter* spp. positive and the prevalence in slaughter age pigs ranges between 53 and 85% (8,9). *Campylobacter coli* from pigs are commonly resistant to macrolides (erythromycin and azithromycin, 43 to 81%) while resistance to quinolones is more variable (ciprofloxacin, 3 to 100%; nalidixic acid, 5 to 100%) (10-17). In North America, swine producers use antimicrobials to treat and prevent disease and to improve growth performance (18,19). Despite this, the relationship between antimicrobial use and resistance in *Campylobacter* spp. has not been described on swine farms. Furthermore, antimicrobial resistance in *Campylobacter* from pigs on Canadian farms has not been described to date. This study's primarily objective was to describe antimicrobial resistance (AMR) in *Campylobacter* spp. from grow-finish pigs on western

Canadian farms (Alberta and Saskatchewan). Additional objectives were to evaluate antimicrobial use as a risk factor for resistance to macrolides and quinolones, and to estimate the variability in resistance within herds. Clustering within herds indicates the potential for herd level interventions to control AMR (20,21).

8.2 Materials and Methods

8.2.1 Herd selection and data collection

The number of herds enrolled, and samples analyzed, were subject to practical and financial constraints. Eight swine veterinarians each recruited two to four study herds. Thirteen herds were in Saskatchewan and seven in Alberta. Herd inclusion criteria included a minimum of 100 sows and enrollment in the Canadian Quality Assurance® (CQA®) Program (22). At selection, the researchers were blind to antimicrobial use practices in the herds.

Each herd was visited between May and September of 2004. During this visit, 20 to 25 pens of apparently healthy grow-finish pigs were identified using a random numbers table. One composite sample, consisting of feces from five pigs, was collected from each of these pens. The herd owner or manager used existing records to complete a survey describing antimicrobial use through feed and water at the production phase level. For every exposure of suckling, nursery, grow-finish pigs and sows in the previous 12 months, the product used, number of pigs, and number of days was recorded. Inventory and management data were also collected (Table 8.1). Antimicrobial use was quantified as the antimicrobial exposure incidence (AEI) and reported for 1,000 or 100,000 pig-days at risk to facilitate interpretation (Equation 1).

Equation 1. Formula for Antimicrobial Exposure Incidence (AEI)

$AEI = [Pigs_E * Days_E] / [Pigs_R * Days_R]$

E = exposed

R = at risk

8.2.2 Isolation and identification

Samples were sent on ice to the Laboratory Services Division, University of Guelph, Guelph, Ontario for *Campylobacter* isolation within 24 h of collection. The isolation protocol was based on two previously reported methods (23,24). From each sample, a 25 g fecal aliquot was homogenated in 225 ml of Rosef's broth. The Rosef's broth contained 10 g Peptone (BD), 8 g lab Lemco powder (Oxoid), 1 g yeast extract (BD), 5 g NaCl (Fisher), 16 ml of Resazurin solution (25 mg of Resazurin (Difco) in 100 ml distilled water) and 984 ml of distilled water. One swab of the homogenated mixture was streaked onto Charcoal Selective Media (CSM; OXOID CM0739 with supplement OXOID SR155) and incubated at 43° C for 48 to 72 h. Selective enrichment was also performed; 625 µl of FBP solution (equal volume mixture of: 10% ferrous sulfate, 10% sodium metabisulfite and 10% sodium pyruvate) was added to the Rosef's broth and incubated at 30° C for 4 h. Following this, 1.0 ml of cefoperazone (Sigma C4292), 1.0 ml of cycloheximide (HP Biomedicals 100183), and 5.0 ml of VTP [0.5 g vancomycin (Sigma V2002), 0.25g trimethoprim (Sigma T0667), 0.035 g polymyxin B sulfate (Sigma P1004) in 1 L distilled water] were added and the broth was re-incubated at 37° C for 2 h followed by 43° C for 44 h. A swab of the enriched broth was streaked onto Mueller-Hinton Blood Agar (MHBA) plates that contained the same antibiotics as above. The MHBA plates were incubated at 43° C for 48 to 72 h. Positive control of *Campylobacter jejuni* ATCC 33560 and negative control of *Escherichia coli* ATCC 25922 were run concurrently. All incubations occurred under microaerophilic conditions (85% nitrogen, 10% carbon dioxide and 5% oxygen).

Typical colonies were selected from the CSM and MHBA plates for darkfield microscopy examination using the wet-mount technique. Those with typical corkscrew motility were subcultured onto MHBA plates and incubated at 35° C for 24 to 48 h to obtain pure cultures. *Campylobacter* identification was based on colonies being Gram-negative, catalase positive, oxidase positive and having a spiral S-shape or sea gull-wing morphology. The *Campylobacter* species was identified through indoxyl acetate and hippurate hydrolysis (25,26). A swab of growth from the MHBA plate was mixed in glycerol peptone (250 ml Glycerol (Neutral) (Sigma), 10g Neo-peptone (BD), 5g NaCl (Fisher S671) and 750 ml distilled water) to create a turbid suspension. Growth from the same plate was collected and inoculated into a cryovial with beads. The glycerol peptone culture and cryovial were stored at minus 80° C.

8.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Laboratory for Foodborne Zoonoses, Guelph, Ontario using the Etest® (AB Biodisk) according to the manufacturer's instructions. Briefly, *Campylobacter* isolates were subcultured onto MHBA plates and incubated overnight at 42° C. All incubations were under microaerophilic conditions (85% nitrogen, 10% carbon dioxide and 5% oxygen). Colonies were suspended in 2 ml of Mueller-Hinton broth to obtain turbidity equivalent to a 0.5 McFarland standard using a MicroScan turbidity meter (Dade Behring Inc., West Sacramento CA*)*. The suspensions were streaked onto Mueller-Hinton Agar plates supplemented with 5% laked horse blood with a sterile swab to create a lawn growth over the entire plate. Pairs of Etest® strips containing ampicillin, azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, spectinomycin, or tetracycline, were applied in antiparallel fashion onto each plate and incubated in a

microaerophilic environment at 37° C for 48 h. Bactericidal antibiotic Etest® strips were read where 100% inhibition intersected with the strip (Table 8.2). If this occurred between marks on the strip, the minimum inhibitory concentration (MIC) was rounded up to the next gradient marking. Bacteriostatic antibiotic strips were read at 80% inhibition / 20% growth (Table 8.2). If this occurred between markings on the strip, the MIC was rounded down. Breakpoints used by the National Antimicrobial Resistance Monitoring System (NARMS) and Canadian Integrated Program for Antimicrobial Resistance Surveillance (Dutil, personal communication), based on the Clinical and Laboratory Standards Institute (CLSI) recommendations for Enterobacteriaceae, were used (27-30). Specifically the resistance breakpoints were as follows: ampicillin ≥32, azithromycin ≥8, chloramphenicol ≥32, ciprofloxacin ≥4, clindamycin ≥8, erythromycin ≥32, gentamicin ≥8, nalidixic acid ≥64, spectinomycin ≥32 and tetracycline ≥16. Spectinomycin breakpoints were based on those previously used in Denmark because CIPARS and NARMS have no established breakpoint (16). Quality control organisms included *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559. Quality control was ensured by incubating these, and a blank control, at 37° C under microaerophilic conditions for 48 h. The *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and blank control were also incubated at 37° C under aerobic conditions for 24 h.

8.2.4 Statistical analysis

As defined by the World Health Organization (WHO), an antimicrobial class refers to "agents with similar chemical structures that exert an effect on the same target in bacteria and may be affected by the same mechanism of resistance" (7). Five classes were represented by a single antimicrobial: ampicillin, chloramphenicol, gentamicin, spectinomycin, and tetracycline.

Two classes had more than one antimicrobial considered because of their importance to human medicine. An isolate was considered resistant to quinolones if resistant to ciprofloxacin or nalidixic acid, and resistant to macrolides if resistant to azithromycin (an azalide), or clindamycin (a lincosamide), or erythromycin (a macrolide).

Data were stored and organized using Microsoft Access (Microsoft Corporation, USA) and descriptive statistics were calculated using Microsoft Excel (Microsoft Corporation, USA). Generalized estimating equations (PROC GENMOD, SAS for Windows version 9.1, SAS Institute Inc., Cary, North Carolina) were used to estimate the prevalence of AMR and examine the association between antimicrobial use and AMR. All models had a binomial distribution, logit link function and adjusted for clustering within herds using an exchangeable correlation structure (20). When necessary, extra-binomial variation was permitted by a scale parameter equal to the square root of the Pearson's Chi-Square divided by the degrees of freedom in the model. Population-average prevalence estimates for resistance to each antimicrobial, and each drug class, were calculated from the intercept (β_0) and 95% confidence intervals (CI) of the null models using the formula $1/[1 + \exp(-\beta_0)]$ (20). Susceptibility results were not stratified by *Campylobacter* species as biochemical speciation is imperfect, and isolates were predominantly *C. coli* (31).

Antimicrobial use variables were investigated as potential risk factors for resistance to macrolides and quinolones, important therapeutic drugs for human campylobacteriosis. Antimicrobial use was categorized into five classes: i) aminoglycosides (including aminocyclitols), ii) beta-lactams, iii) macrolides (including lincosamides and pleuromutilins), iv)

sulfonamides, and v) tetracyclines (7,32). Antimicrobial use was then stratified by the production phase exposed.

The following model building strategy was used for each resistance outcome. The unconditional association between each antimicrobial use variable and resistance was determined. Outcomes were modeled using the number of resistant isolates in the herd (numerator) over the number of isolates tested in the herd (denominator). Predictor variables were modeled as continuous if the AEI was greater than zero in five or more herds. The associations between antimicrobial use, measured as a continuous variable, and resistance were examined for linearity by graphically assessing the effect estimate for each quartile against the log odds of the outcome. Antimicrobial use variables associated with the outcome in a non-linear manner were categorized as no use, low use (0.1 to 500 treatments per 1000 pig-days) and high use (>500 treatments per 1000 pig-days) because this categorization best fit the data. Variables used in less than five herds were collapsed to 'any use' versus 'no use' (dichotomous). Variables reported in only one herd were not examined.

Antimicrobial use variables with an unconditional $P < 0.2$ were included in the full model. Manual step-wise backward selection was used to develop a main effects model, retaining only variables significant at $P < 0.05$. Variables removed from the full model were re-introduced into the main effects model individually; this prevented inappropriate removal due to confounding. Two-way interactions were considered between significant variables within a production phase and were retained, along with their main effects, if $P < 0.05$. Eight management variables were examined for potential confounding (Table 8.1). These were

retained, along with their main effects, if their inclusion altered a parameter estimate by more than 25%. Residuals of the final models were examined visually for outliers (20). The association between each variable of interest and the outcome was reported as an odds ratio (OR=expβ) with 95% CI (20).

A second modeling approach estimated the extent AMR clustered within herds. The variance at the herd level was determined using restricted maximum likelihood estimation. Models had a binomial distribution, logit link function and modeled the outcome as the proportion of resistant isolates in a herd in the numerator and number of isolates tested in the denominator. Under-dispersion was accounted for by allowing random variation at the lowest level (21). The restricted generalized iterative least-squares (RIGLS) algorithm (MLwiN version 2.0r, Centre for Multilevel Modelling, Institute of Education, University of London, London, England) was used and second-order penalized quasi-likelihood (PQL-2) estimates were reported (21,33). Intra-class correlation coefficients (ICC) were approximated by the latent variable approach (20,21,33). Specifically, the herd variance was divided by the total variance after fixing the error variance at $\pi^2/3$.

8.3 Results

8.3.1 Prevalence of *Campylobacter* spp. and antimicrobial susceptibility

Campylobacter were isolated from 438 of 444 pooled fecal samples. Of the positive samples, 86.5% were *C. coli* (379), 2.3% were *C. jejuni* (10), 0.9% were *C. lari* (4) and 10.3% (45) could not be speciated because the lawn growth was too weak to perform hippurate hydrolysis and indoxyl acetate tests. Antimicrobial susceptibility testing was performed on 405 isolates (average; 20 per herd). These included all of the *C. coli*, *C. jejuni* and *C. lari* and 12

unspeciated isolates. The remaining 33 unspeciated samples also had lawn growth too weak for susceptibility testing.

The prevalence of resistance to individual drugs ranged from 0 to 71% (Table 8.2). The highest prevalence of resistance was to clindamycin followed by azithromycin and erythromycin (Table 8.2). Concurrent resistance to these three drugs was the most common pattern identified (Table 8.3). With the exception of three isolates, which were resistant to clindamycin but susceptible to erythromycin and azithromycin, isolates resistant to any one of these drugs were resistant to all three. Therefore, although the WHO classifies clindamycin separately from the macrolides, these drugs were grouped as one resistance class for the purpose of this study. Cross-resistance was not complete between ciprofloxacin and nalidixic acid. Thirty-five isolates were resistant to both drugs, two were resistant to ciprofloxacin only, and eleven were resistant to nalidixic acid only. Hence, 14.8% (95% CI, 6.5 to 30.2) of the isolates were classified as quinolone resistant. Twelve percent (95% CI, 5.5 to 24.0) of the isolates were susceptible to all drugs while 64.0% (95% CI, 47.1 to 78.0) were resistant to drugs in two or more classes. Three percent (12) of the *Campylobacter* spp. were resistant to drugs in four of the seven antimicrobial classes.

8.3.2 Antimicrobial use and resistance

In every herd, antimicrobials were administered through feed and/or water to groups of pigs in the previous 12 months. Suckling pigs were exposed to antimicrobials in 10 herds, nursery pigs in all 20 herds, grow-finish pigs in 15 herds, and sows in 8 herds. Seven producers used sulfonamides, 9 used aminoglycosides, 9 used beta-lactams, 15 used tetracyclines and 18

used macrolides (Table 8.4). No producers reported using quinolones, chloramphenicol or florfenicol.

Campylobacter spp. resistance to macrolides and quinolones were each associated with one antimicrobial use variable. The odds of resistance to a macrolide increased 1.3 times (95% CI, 1.0 to 1.6; $P = 0.049$) for every 100,000 pig days macrolides were administered to nursery pigs. In contrast, the odds of resistance to a quinolone were 8.9 times higher (95% CI, 1.3 to 61.9; $P = 0.03$) in *Campylobacter* spp. from herds with no beta-lactam use in grow-finish pigs compared to *Campylobacter* spp. from herds with exposure. Management variables were not important confounders in either relationship (Table 8.1).

8.3.3 Within herd variability in antimicrobial resistance

All of the isolates from individual herds were often either susceptible or resistant to a drug class, providing evidence that AMR clustered within herds (Table 8.5). This was most notable for macrolide resistance as only six herds had a mixture of susceptible and resistant isolates. The extremely high intra-class correlations (ICC) described just how similar the AMR was for isolates from the same herd (Table 8.5). Even tetracycline, the resistance with the lowest ICC, showed very strong clustering within herds.

8.4 Discussion

Many of these *Campylobacter* spp., isolated from grow-finish pigs on farms in western Canada were resistant to macrolides and quinolones. To date, the role of pork in foodborne campylobacteriosis is unclear because the source of human infections is rarely identified and risk factor studies seldom consider *C. coli* independently (6,34,35). Similarly, the public health

significance of AMR is also unmeasured because tracing resistance in animals to disease in people is difficult (6,35,36). However, *C. coli* can rank among the top four causes of human enteric infections (37). When treatment is required, macrolides and quinolones are first-line therapies (5-7). Thus given *Campylobacter's* zoonotic potential, the frequency of resistance in these isolates is concerning.

It is also concerning that only 12% of these isolates were susceptible to all ten drugs tested. Resistance was largely attributable to the macrolides and our findings are consistent with other North American and European reports (10-17). *Campylobacter coli*, and particularly *C. coli* from pigs, typically have a higher prevalence of resistance to macrolides than *C. jejuni* (12-16,38). Although the reasons for this remain speculative, it is notable that macrolides are used extensively in pigs (18,19,39).

The 10% observed resistance to ciprofloxacin was interesting considering no study herds reportedly used quinolones. The frequency of resistance was similar to other North America findings (1 to 11%) (15,17,38,40) and lower than reported in Europe (28 to 100%) (10,12-14). These isolates' susceptibility to chloramphenicol and gentamicin also corresponded with previous North American reports (0 to <5%) (9,15,17). To date, only European studies have considered spectinomycin resistance and our data fell in the middle of these previous reports (4 and 94%) (10,16). Although considerably more ampicillin resistance has been described in Spain (66%), our findings were similar to other Canadian and European studies (6 to 20%) (11,13-16). Ampicillin and spectinomycin resistance were of interest because beta-lactams and spectinomycin are used in Canadian pigs (18,39). Finally, the frequency of resistance to

tetracycline was lower than other North American reports (68 to 71%) (15,17). Interestingly, it was also lower than that reported from antibiotic-free farms in the United States (56%) (9).

Resistance to multiple antimicrobial classes was described, rather than resistance to multiple drugs. This more accurately depicted the extent of multiresistance because isolates had almost complete cross-resistance within the macrolide and quinolone classes. Comparing the level of multiresistance between studies is hindered by differences in the antimicrobials tested. However, the 64% resistance to two or more drug classes was much closer to the level of multiresistance described in France and Belgium than the 4% resistance to three or more drugs reported from antibiotic-free swine herds in the United States (11,14,41). *Campylobacter* from conventional swine herds have shown significantly more multiple resistance than those from herds with antibiotic free production (9). Likewise, *Campylobacter* spp. from herds with lame and ill-thrift pigs (where herd health variables reflected antimicrobial use) had substantially higher odds of being multiple resistant (42). Considering these previous reports, it was important to consider antimicrobial exposures as risk factors for resistance.

High level *Campylobacter* resistance to erythromycin, as seen in these isolates, is predominantly due to a mutation in the 23S rRNA gene (43,44). Thus, the association between macrolide use and resistance suggests macrolide exposure selected for mutated bacteria. Although these *Campylobacter* spp. were from grow-finish pigs, antimicrobial exposure in nursery pigs was the significant risk factor. Observational and experimental studies support that antimicrobial use in one production phase can affect AMR in pigs from another phase (45-47). This finding may be of great clinical importance because macrolides are used extensively in

Canadian pigs (18,19). We feel it is crucial that swine producers and veterinarians know antimicrobial exposure in young pigs may have food safety repercussions.

Enrofloxacin exposure can increase *C. coli* resistance to quinolones (48). However, direct selection does not explain these isolates' resistance because study herds reported no quinolone exposure. Others have also found ciprofloxacin resistant *Campylobacter* in swine herds without quinolone exposure, and even in herds raising antibiotic-free pigs (9,41,42). This project identified one antimicrobial use risk-factor for quinolone resistance; administering beta-lactams to grow-finish pigs appeared protective. We propose three possible explanations for this finding. First, it may be a true association reflecting an undefined, antagonistic relationship between quinolone and beta-lactam resistance genes. Second, considering the strong association and the low exposure incidence in the herds with grow-finish beta-lactam exposure, this variable may have acted as a proxy for another common, but undefined, risk factor. Third, the association could be spurious. Although this seems unlikely given the bounds of the confidence interval, future studies should re-investigate this relationship.

Strong similarities were found between individual drug resistances within herds. This could indicate two things: risk factors for resistance may have been present and pervasive in some herds but not present in others or clustering could be due to resistant clones. The clonal hypothesis is unlikely considering resistance phenotypes varied within herds (data not shown). For example, although macrolide resistance was highly similar within a herd the macrolide resistant isolates carried different additional resistances. Additionally, others have found extensive genetic diversity in resistant *Campylobacter* in swine herds (49,50). Future on-farm

studies might attempt to explain this uniformity within herds. Meanwhile, clustering must be considered in study design and statistical analyses.

This study's primary weakness was the small number of herds in this convenience sample; a limitation common to pilot studies. The authors had no knowledge of the antimicrobial use practices in herds at the time of enrollment. However, to ensure adequate antimicrobial use records, herds had to be enrolled in the CQA® program (22). Although this could have influenced antimicrobial use practices, it is unlikely to have biased results because at the time of the study more than 98% of herds in Alberta and Saskatchewan participated in this program (personal communication Sarah Turner, Alberta Pork and Harvey Wagner, Sask Pork). Repeating this study in a larger number of randomly selected herds would improve the ability to extrapolate prevalence results. It would also make risk factor estimates more precise and might allow consideration of individual drugs rather than antimicrobial classes.

Extrapolating these data beyond the study herds should be done with caution. Likewise, comparing these data to other publications should be done cautiously because isolation techniques, antimicrobial susceptibility testing methods and breakpoints can affect results (51). Presenting our data with other North American reports provides a regional context (9,15,17,38,41). European studies were referred to because of extensive reporting on *Campylobacter* AMR (10-14,16). However, we do not suggest that differences in AMR are soley due to variations in antimicrobial use because geography, temporality, seasonality and husbandry were not considered.

This project described the prevalence of antimicrobial resistant *Campylobacter* spp. from 20 swine herds in Alberta and Saskatchewan, Canada. It identified a dose-response relationship between macrolide use and resistance. This study was cross sectional and could not definitively prove that antimicrobial use caused the observed resistance. However, in light of previous evidence, the study findings support a causal link between antimicrobial exposure and resistance in pigs (47,48,52). Continued research may generate sufficient knowledge to control AMR on-farm. As this would ultimately mitigate concerns about food safety from antimicrobial resistance in pork, we strongly advocate for continued on-farm AMR research.

8.5. References

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Table 8.1 Management variables considered as potential confounders of the relationship between antimicrobial use and resistance (N=20 herds).

Level	Variable		Herds
Herd	Purpose for which animals were sold	breeding stock	7
		slaughter only	13
	Sites farrow-to-finish herd located on	one	15
		two	2
		three	3
Grow-finish	All-in-all-out by room or barn	yes	9
		no	11
	Pens emptied between batches	yes	12
		no	8
	Manure removed between batches	yes	10
		no	10
	Pens washed and disinfected between batches	yes	8
		no	12
	Times barn washed in previous 12 months	mean (*S*)[a]	3 (3.7)
	Days pens empty between batches	mean (*S*)	1 (1.7)

a: *S* represents standard deviation

Table 8.2 Frequency of resistant *Campylobacter* spp., percent prevalence of resistance adjusted for herd-level clustering with 95% confidence intervals, and MIC_{50} and MIC_{90} (n = 405).

Antimicrobial [a]	Frequency	Prevalence	95% CI	MIC_{50}	MIC_{90}
Clindamycin [s]	300	71.3	51.6 - 85.3	256	256
Azithromycin [s]	297	70.5	50.8 - 84.7	256	256
Erythromycin[s]	297	70.5	50.8 - 84.7	256	256
Spectinomycin [s]	190	45.6	28.6 - 63.7	8	1024
Tetracycline[s]	145	35.1	23.7 - 48.5	0.75	256
Ampicillin [s]	57	14.8	7.5 - 27.2	6	32
Nalidixic Acid [c]	46	14.3	6.2 - 29.8	8	256
Ciprofloxacin [c]	37	10.1	3.3 - 26.6	0.125	2
Chloramphenicol [s]	7	1.9	0.3 - 12.2	3	16
Gentamicin [c]	0	0	0.0 - 0.0	0.5	0.75

a: Superscript ‘s’ denotes bacteriostatic antimicrobials and ‘c’ denotes bacteriocidal.
MIC (µg/ml); MIC_{50} and MIC_{90}indicates the MIC at which 50% and 90% of the isolates were inhibited respectively

Table 8.3 Frequency of the ten most common antimicrobial resistance phenotypes observed in *Campylobacter* spp. isolated from grow-finish pigs (n = 405).

Resistance phenotype	Frequency	Percent
AZM-CLI-ERY	71	17.5
AZM-CLI-ERY-SPT	69	17.0
AZM-CLI-ERY-TET	51	12.6
AZM-CLI-ERY-SPT-TET	46	11.4
AMP-AZM-CLI-ERY-SPT	26	6.4
TET	15	3.7
CIP-NAL	13	3.2
AZM-CIP-CLI-ERY-NAL-SPT	12	3.0
AMP-SPT-TET	7	1.7
AMP-AZM-CIP-CLI-ERY-NAL-SPT	6	1.5

AMP, ampicillin; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; NAL, nalidixic acid; SPT, spectinomycin; TET, tetracycline

Table 8.4 Frequency of herds administering antimicrobials to each production phase (N = 20 herds), and antimicrobial exposure incidence per 1000 pig-days in herds reporting an exposure incidence greater than zero.

Antimicrobial Class	Production phase	# of Herds reporting use	AEI in herds with any use	
			Median	IQR[a]
Aminoglycosides	Suckling	3	700	517 - 850
	Nursery	8	272	137 - 501
	Grow-finish	0	-	-
	Sow	0	-	-
Beta-lactams	Suckling	3	286	238 - 310
	Nursery	8	210	176 - 291
	Grow-finish	3	11	6 - 17
	Sow	1	64	-
Macrolides	Suckling	8	310	228 - 572
	Nursery	17	528	411 - 974
	Grow-finish	15	714	160 - 977
	Sow	3	34	18 - 167
Sulfonamides	Suckling	3	286	238 - 310
	Nursery	6	143	126 - 191
	Grow-finish	1	24	-
	Sow	0	-	-
Tetracyclines	Suckling	5	333	273 - 381
	Nursery	13	226	220 - 508
	Grow-finish	1	725	-
	Sow	5	37	16 - 185

a: IQR = Interquartile range presented for herds with treatment incidence > 0

Table 8.5 Distribution of *Campylobacter* spp. resistance to each antimicrobial class within herds, herd variance and intra-class correlation between isolates within herds (N = 20 herds, n = 405 isolates).

Representative of Antimicrobial Class*	Number of herds		Herd Variance (SE) [a]	Intra-class Correlation
	Completely susceptible	Completely resistant		
Ampicillin	9	0	6.8 (3.5)	0.67
Chloramphenicol	19	0	-	-
Gentamicin	20	0	-	-
Macrolides	4	10	14.9 (11.8)	0.82
Quinolones	8	1	12.4 (9.1)	0.79
Spectinomycin	6	3	8.5 (4.0)	0.72
Tetracycline	6	0	3.8 (1.4)	0.53

* Variance and ICC only estimated for *Campylobacter* resistances observed at >5% prevalence to ensure convergence in RIGLS PQL-2

a: Standard errors of estimate in parenthesis

CHAPTER 9
CONCLUSIONS

This thesis described the antimicrobial resistance (AMR) of gram negative enteric bacteria from healthy pigs and investigated the relationship between resistance in *E. coli* and *Campylobacter* and antimicrobial exposure in swine herds. Antimicrobial resistant bacteria from livestock are a food safety hazard; resistant bacteria from animals can pass through the food processing chain and infect consumers (1-5). Pigs commonly receive antimicrobials and these exposures contribute to antimicrobial resistance (AMR). However, the extent of antimicrobial use and the relationship between antimicrobial exposure and resistance had not previously been described in western Canadian swine herds. This study was conducted in Alberta and Saskatchewan as together, these provinces produce 20% of Canada's market hogs (6). Despite this sizable industry, on-farm AMR had only been described in *Salmonella* from grow-finish pigs (7). In this concluding chapter, the key findings of this thesis are presented and the strengths and limitations of the project are considered. As is often the case in research, our finding generated many new questions. Therefore, future research directions are also presented.

To address the objectives of this thesis, a cross-sectional study was conducted in a convenience sample of 20 swine herds. The antimicrobial exposure incidence in each herd was described from existing records and fresh fecal samples were collected from pens of healthy grow-finish pigs. In half of the herds, additional samples were collected from nursery pigs and sows. This allowed AMR in *Salmonella* to be compared across

production phases. The AMR phenotypes of *Salmonella, Escherichia coli,* and *Campylobacter* were described and a subset of *E. coli* was tested for resistance genes and virulence factors.

Chapter 3 described antimicrobial use in study herds. This project differed from previous descriptions of antimicrobial use in Canadian pigs because the antimicrobial exposure incidence (AEI) per pig-day was estimated from existing on-farm records. The AEI through feed, water and injection of pigs in the suckling, nursery, grow-finish and sow production phases was described for a 12 month-period. Previously, this measure had only been used to estimate the injectable exposure incidence from prospectively maintained records (8).

These study herds used similar antimicrobials and routes of administration as herds in Alberta, British Columbia, Ontario and the United States (8-12). Almost all herds used antimicrobials in feed, approximately half used water medications, and nursery pigs were the most commonly medicated production phase. On any day, the probability of in-feed exposure was 0.8 for a nursery pig and 0.3 for a grow-finish pig. Describing antimicrobial use as an incidence showed the extent that exposure varied among herds. Most notably, in-feed medication of grow-finish pigs varied from 0 to 1000 exposures per 1000 pig-days. Although different disease pressures explained some of this variation, much was for growth promotion. This may be a target for judicious use campaigns: Grow benefits decline as pigs age and Scandinavian producers experienced

minimal disease problems in grower and finisher pigs following bans of growth promotion drugs (13-15).

This study demonstrated that on-farm records maintained by producers enrolled in the CQA® program provided sufficient information to describe the AEI of groups of pigs. Exposure incidences allow relatively small changes in antimicrobial use, such as targeted medication at high risk groups, or shortened exposure durations, to be recognized. If future studies and monitoring programs report antimicrobial exposure incidences, rather than qualitative data, subtle decreases in drug use will be captured. Future research into management practices on farms with minimal drug use might identify strategies that eliminate unnecessary antimicrobial exposures. Widespread implementation of such strategies could decrease antimicrobial use in Canadian pigs while potentially improving profits of swine producers.

In the fourth chapter, the frequency and patterns of AMR in *Salmonella* spp. were described. This thesis expanded our knowledge of on-farm AMR by testing *Salmonella* from apparently healthy grow-finish pigs in all herds, plus sows and nursery pigs in half of the study herds. Few studies have collected resistance data from animals distant from market; a study in North Carolina collected samples from multiple phases but did not compare resistance across phases, while another study compared resistance across phases but only studied three herds (16,17). Thus, describing AMR resistance in nursery pigs, grow-finish pigs and sows was novel. The differences observed between production

phases indicate there is much to learn about the development, persistence and spread of resistance within modern swine barns.

The overall, or pooled, frequencies of resistance in these *Salmonella* isolates were similar to previous reports from healthy grow-finish pigs on farms in Alberta and from pigs sampled at abattoirs across Canada (9,18). However, differences were found between phases. *Salmonella* isolates from sows had less resistance to individual antimicrobials, and were more likely to be pansusceptible, than the isolates from the other phases. In contrast, *Salmonella* spp. from nursery pigs had more resistance to most of the antimicrobials tested, and were more likely to be multiresistant than isolates from the other phases. A tendency for young pigs to have increased rates of resistance has been reported in *E. coli*, but has not been well described in *Salmonella* (19,20). Others have speculated resistance may be increased due to physiological differences in the gut flora but increased resistance from antimicrobial use is also plausible considering the high exposure rates in these nursery pigs. Future studies should aim for sufficient power to account for confounding by serotype and evaluate on farm risk factors, particularly antimicrobial use. This would enable researchers to distinguish between normal gut ecology and on-farm management practices as causes for increased AMR in young pigs.

Describing different resistance rates in each production phase has implications for mitigating resistance in pigs. Identifying less resistance in sows is encouraging because suckling piglets should be exposed to less resistant bacteria. The increased resistance in nursery pigs suggests the nursery phase may be a target for control measures. Finally,

finding lower resistance in grow-finish pigs compared to nursery animals confirms that resistance is dynamic within barns; future research should seek to identify and exploit the factors that cause any decreases in resistance. Future studies will need to investigate not only what but when interventions to control AMR are most effective.

Understanding *Salmonella* AMR on farms is complicated by the relationship between serovar and resistance (17,21). Our study found certain resistance phenotypes occurred only in certain serovars and those serovars appeared linked to farm. Molecular studies could clarify the clonal relatedness between isolates within herds; these studies would be valuable as interventions to prevent the spread and persistence of clones may differ from those aimed to prevent the development of resistance. Studies investigating risk factors for *Salmonella* AMR require many isolates from numerous herds to differentiate potential herd and serovar effects. The relatively low number of isolates and herds in this study precluded antimicrobial exposure risk factor analyses.

Antimicrobial resistance in *E. coli* was the principal focus of this thesis; three chapters were devoted to *E. coli* antimicrobial resistance phenotypes, genotypes and risk factors. *Escherichia coli* AMR phenotypes were described as the prevalence of resistance to each drug and multiple drug resistance patterns, as well as by estimating odds ratios between resistances. Overall, the frequency of pansusceptible isolates (21%) was similar to other on-farm studies in Canada (11,22). Encouragingly, all isolates were susceptible to ciprofloxacin and ceftriaxone. These drugs are considered of critical importance to human health due to the consequences of resistant human pathogens (23-25). Of concern,

one isolate was resistant to nalidixic acid, an indicator of emerging fluoroquinolone resistance (26). Similarly, one isolate was resistant to the third-generation cephalosporin ceftiofur. Although infrequent in this study, on-farm monitoring in the United States has found 1.5% of *E. coli* were resistant to ceftiofur (27,28). Thus, a low prevalence of ceftiofur resistant *E. coli* and more common ceftiofur resistant *Salmonella* from United States farms should remind Canadian authorities to be vigilant for emerging resistance to this drug (28).

As found in the *Salmonella* isolates, the *E. coli* isolates were most commonly resistant to tetracycline, sulfamethoxazole and streptomycin. North American surveillance programs have also found the ranking of resistances to be similar between these two Enterobacteriaceae (28). Considering that these bacterial species can share resistance genes, the herd-level prevalence of resistance in *E. coli* might predict resistance in *Salmonella* (29,30). Resistance to seven different drugs was considered but only two associations were significant. Thus, *E. coli* was not a very effective on-farm sentinel for *Salmonella* AMR in this study population. Considering that *E. coli* has multiple resistance genes for each of the considered resistance phenotypes, it is still possible the prevalence of AMR genes in *E. coli* could predict AMR genes in *Salmonella*. Conversely, if serotype and resistant clones in *Salmonella* were accounted for, phenotypic resistance in *E. coli* could yet prove to be a useful sentinel for AMR in *Salmonella* in swine farms.

Most parametric statistical tests assume data are independent (31). However, *E. coli* from one herd could be more similar than *E. coli* from different farms because pig-to-pig contact facilitates AMR gene transmission, animals have similar antimicrobial exposures, and herd-mates are raised under the same barn design, hygiene and management. Failure to account for clustering can result in type I error (31). So multi-level models are used to provide accurate assessments of confidence levels and statistical significance. Additionally, these models indicate where unexplained variability remains in hierarchical datasets. Interventions aimed at hierarchal levels with more variation are more likely to be effective (31,32). The similarity in resistance within herds suggests farm-level interventions could help manage AMR in *E. coli*. Although based on a small dataset, we found little clustering at the veterinarian level. This suggests that interventions targeted at swine veterinarians would be markedly less effective.

The molecular basis for multiple drug resistance and co-selection in bacteria are well understood. Yet in reality, it is difficult to predict if a given antimicrobial exposure will select for unrelated, but linked, AMR genes under field conditions. To provide insight to this problem, we investigated which resistance-phenotypes were more likely to occur together. In these isolates, every drug-resistance phenotype was significantly associated with at least two other drug-resistances. Remarkably, the odds of sulfamethoxazole resistance increased significantly in the presence of every drug-resistance that we considered. This is of immediate importance to the swine industry because the widespread use of sulfonamides could select for resistance to many unrelated drugs. The numerous associations between resistance phenotypes in this study re-

enforces the take-home message of this thesis: using drugs considered 'older' or 'of lesser importance' to human medicine is not a risk free practice (23-25). For example, penicillin use in grow-finish pigs was associated with increased odds of trimethoprim-sulfamethoxazole resistance in *E. coli*.

Chapter 6 described the diversity of genes carried by a subset of the *E. coli* described in Chapter 3. Prior to this study, the AMR genes carried by porcine commensal *E. coli* had only been described in isolates from Ontario. The isolate selection was purposive; isolates were chosen to avoid clustering within pens and ensure a minimum number of isolates showed phenotypic resistance to seven antimicrobials. This improved the power of the numerous univariate analyses but meant the frequency of AMR genes did not accurately reflect the prevalence of resistance genes in these herds. Despite this limitation, identifying *sul3* as the most common sulfonamide resistance gene was notable. The *sul3* gene was first described in pigs from Switzerland in 2003 (33). Although this gene is recently emerged, it has since been reported in many European countries as well as the United States, Ontario, and now Alberta and Saskatchewan (34-38). Such rapid emergence and spread demonstrates the global nature of antimicrobial resistance.

No chloramphenicol resistance genes were identified in this study. The absence of *cmlA*, *floR* and *cat1* in these isolates indicates that the chloramphenicol resistance in this population differs from porcine *E. coli* in Ontario and Oklahoma (39,40). This unexpected finding illustrates the value of describing AMR by both phenotype and genotype. Although phenotype reflects the risk posed by resistant bacteria on-farms, and

the expected impact of antimicrobial exposure on bacteria, the resistance genotype can distinguish between apparently equivalent resistances in different regions, farms or animals. This information can then provide insight into the evolution of resistance. Chloramphenicol was banned in Canadian livestock in 1985, and so resistance presumably persists due to co-selection with other resistance and virulence genes (34,39-42). If the chloramphenicol genes and their linkages were described in *E. coli* from western Canadian pigs, it might clarify how chloramphenicol resistance in persisting in *E. coli* in western Canadian pigs.

The associations between AMR genes generated hypotheses about gene-linkages. Two sets of genes were interrelated: one set contained *aadA1*, *sul1*, and *tetA* and the other consisted of bla_{TEM}, *strA – strB*, *sul2* and *tetB*. Interestingly, each set contained a streptomycin, sulfamethoxazole and tetracycline resistance gene. So theoretically, amoxicillin use would select for streptomycin, sulfamethoxazole and tetracycline resistance in *E. coli* carrying the second set of genes but not the first. The associations between AMR genes matched associations between the phenotypes for each of the genes. As odds ratios between AMR genes can predict gene linkages (39), this suggests the odds ratios between AMR phenotypes could predict co-selection. If this approach is validated, analysis of nationally representative AMR data might identify antimicrobial exposures that are increasing resistance to important, but unrelated, drugs. The potential for this to improve our understanding of resistance makes validating this hypothesis among the most important research needs identified in this thesis.

The associations between AMR genes, and between AMR and virulence genes, were substantially different from reports in pathogenic *E. coli* (35,39,43). This difference suggests resistance gene linkages in *E. coli* are serotype, or pathotype, specific. Although the lack of associations between resistance and virulence genes could be due to low study power, the relationships could also be specific to certain *E. coli* pathotypes. So while the conclusions of this study cannot be extrapolated to pathogens or specific serotypes of *E. coli*, our findings suggest that antimicrobial use does not select for virulence factors in generic *E. coli* from healthy pigs.

Chapter 7 described the associations between phenotypic AMR in *E.* coli and antimicrobial use through feed and water. Six of the seven resistance outcomes considered had at least one significant antimicrobial use predictor. In five, the odds of resistance increased per pig-day of exposure. To my knowledge, this description of dose-response relationships between antimicrobial exposure and resistance is unique. These results suggest that if eliminating antimicrobial exposure is undesirable for animal welfare reasons, or impractical for financial reasons, decreasing antimicrobial use may be still be beneficial. While causal inferences cannot be drawn from cross-sectional studies, dose-response relationships support a causal role for antimicrobial use in resistance (31).

Assuming a causal relationship, the findings of this study indicate that three biological mechanisms contributed to the associations between antimicrobial use and resistance reported in this paper. Direct selection explained associations between antimicrobial resistance and exposure to the same drug: an example was tetracycline use

(along with oxytetracycline and chlortetracycline) in nursery pigs was a risk factor for tetracycline resistance. Cross-resistance best explained associations between a resistance phenotype and exposure to a related drug, as occurred with sulfonamide use in nursery pigs and sulfamethoxazole resistance (although many sulfonamides are licensed for use in Canadian pigs, sulfamethoxazole is not among these) (27). Finally, co-selection was the biological explanation for the associations between resistance and exposure to an unrelated drug, as occurred in four models. Interestingly, macrolide exposure was a significant predictor in all of these models. Macrolides were the most commonly used antimicrobial class in these herds. Therefore, communications with the swine industry should emphasize the concept of co-selection and the possible implications from macrolide use (9,10,12).

Campylobacter is the number one reported cause of foodborne bacterial infection in Canadians (44). The final chapter of this thesis described the antimicrobial resistance of *Campylobacter* spp. recovered from grow-finish pigs on the study farms. This was the first description of AMR in *Campylobacter* spp. from pigs on Canadian swine farms, and included resistance to the first choice treatments for campylobacteriosis in humans, the macrolides and fluoroquinolones. Although these resistances have been reported in other regions of North America, no published studies have investigated antimicrobial use risk factors (28,45-48).

Campylobacter coli was the predominant *Campylobacter* species identified on these farms, and more than 70% of the isolates were resistant to azithromycin,

clindamycin and erythromycin. *Campylobacter coli* are more commonly resistant to macrolides than *C. jejuni.* Additionally, *C. coli* from pigs are more commonly resistant to macrolides than *C. coli* from other species (46,47,49-53). This is concerning considering the extensive use of macrolides in pigs (9,10,12). In this project, macrolide resistance was associated with macrolide exposure of nursery pigs. This finding, in combination with the associations between macrolide exposure and AMR in *E. coli*, re-iterates the need for swine producers to eliminate unessential macrolide use.

Fluoroquinolones are a common antimicrobial treatment for undifferentiated diarrheas in people, which are often caused by *Campylobacter* infection (54,55). Despite the absence of reported quinolone use in study herds, 15% of isolated *Campylobacter* were resistant to a quinolone. Others have also reported quinolone resistance in herds with no quinolone exposure, including herds with no antimicrobial use (48,56). Considering these reports, it was imperative that we investigate farm level risk factors for this resistance. Interestingly, only one predictor of quinolone resistance was found; beta-lactam use in grow-finish pigs appeared protective. However, this negative association does not give any indication of what is causing quinolone resistance to develop or persist, and so this question remains for future studies.

The extreme within-herd similarity in resistance among *Campylobacter* isolates was unforeseen when this project was designed. The intra-class correlations ranged from 0.5 for tetracycline resistance to 0.8 for macrolide resistance. Such clustering within herds indicates great potential for on-farm interventions to mitigate AMR in

Campylobacter (31,32). However, it also means the power of our risk-factor analysis was diminished because the number of independent observations was closer to 20 (the number of herds) than 405 (the number of isolates). Future studies should consider this clustering in their sampling strategy and collect samples from as many herds as possible.

This project was initially developed as a pilot for those developing on-going monitoring programs for antimicrobial use and resistance on swine farms. As is inherent with pilot projects, the number of study herds enrolled, samples collected, and isolates tested were limited. The relatively small number of participating herds impeded our ability to describe the association between antimicrobial resistance and exposures to individual drugs. It also limited the power of the risk-factor analysis for *Campylobacter* resistance. Herds were not randomly selected; rather veterinarians nominated herds based on our enrollment criteria, including knowledge of the *Salmonella* status and willingness to participate in on-going research. Thus, the prevalence estimates reported in this thesis should not be directly extrapolated to other herds in these provinces. However, there was minimal evidence that the herd selection biased our findings; the three factors affected by selection, the *Salmonella* status, herd size, and veterinary practitioner, were rarely associated with any resistance outcomes. Finances also limited the number of *E. coli* isolates tested for AMR genes and virulence factors. The purposive isolate selection ensured a minimum frequency of resistance to the antimicrobials of interest and thus increased the chances of identifying resistance genes. This was a compromise as it improved the power of the univariate analyses but precluded estimating the prevalence of resistance and virulence genes in the study herds.

The findings of this thesis generated many new research questions, but three dominate. First, the differences between antimicrobial resistances in *Salmonella* from nursery pigs, grow-finish pigs and sows raised questions about the dynamics of resistance within herds. Longitudinal studies, which follow batches through the production chain, might identify times when pigs are at risk for acquiring resistance or practices associated with resistance. Just as importantly, management practices associated with declining resistance might also be found and lead to interventions.

Secondly, the parallel between significantly associated pairs of resistance genes and resistance phenotypes in *E. coli* was intriguing. Confirming that co-selection can be predicted by odds ratios between resistance phenotypes would provide veterinarians with a simple tool to consider how their prescribing behavior affects AMR. This could immediately influence antimicrobial use in Canadian agriculture.

Finally, on-farm food safety programs rarely consider the cost:benefit ratio of their recommendations. Resistance has fallen in Denmark since the ban on growth promoters but producers have also experienced an estimated 1.0 € increase in the cost of production from birth to slaughter (14). As differences exist between Canadian and Danish swine production, future studies must evaluate the economics of metaphylactic and prophylactic antimicrobial use in Canadian pork production. This data could then be considered in light of predicted benefits from changes in AMU: such change may be possible as indicated by the highly variable AMU in these study herds. Odds ratios will

never speak to producers with the same intensity as dollars and cents. Veterinary epidemiologists should investigate all aspects of appropriate antimicrobial use, including economics. Should minimal drug use be shown to be economical, authorities and producers would share a common goal.

This thesis adds a few pieces to the complex puzzle of antimicrobial resistance. Undoubtedly, it will be years before we will fully understand the food safety risks from agricultural antimicrobial use. However considering the dearth of new antimicrobials under development for human or veterinary medicine, and the potential for increased resistance to those currently used, both the medical and veterinary communities should follow John Snow's bold example and take action before this problem is fully understood (57). Swine producers must use be educated about co-selection: Using older antimicrobials or drugs unimportant to human medicine is not risk-free. Until science defines which antimicrobial use practices create the greatest food-safety risks, antimicrobials must be used as judiciously as possible. Not only will this minimize any contribution to this public health crisis, it will remove ammunition from calls for restrictions or bans of agricultural antimicrobial use.

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APPENDIX A
ANTIMICROBIAL USE DATA COLLECTION TOOLS

A.1 Instructions for Antimicrobial Use Data Collection

1. Overall Antibiotic Use on Farm
    a. Go through form A stressing that you are asking about the last 12 months.
    b. Note any categories that are different from never
    c. For those categories you will need to go to the next level

2. Feed Medication Use
    a. Copy "RATIONS USED ON FARM" from CQA® records for ALL farms
    b. Complete Form B for any farm which answered different from never on form A for questions 1, 4, 7, 10, 13, 16 (questions relating to feed medication)
    c. NOTE: The questions that you can NOT obtain answers from CQA® form are italicized.
    d. Ensure data is complete for suckling and nursery animals.

3. Water Medication Use
    a. There is No CQA® record for water medication
    b. Complete Form C for any farm which answered different from never on Form A for questions 2,5,8,11,14,17 (questions relating to water medication)

4. Injectable Antibiotic Use
    a. Copy "Pen or Individual Treatment Records For All Pigs Beyond the Weaning Phase" CQA® form
    b. Copy "Medication and Vaccine Usage Plan On Farm" CQA® form
    c. Ensure data is complete each production phase. If not, complete form D

5. Numbers of pigs
    a. Complete form E

6. Duration in each production phase and average weight at transfer
    a. Complete form F

A.2 Overview of Antimicrobial Use

Herd Id: _____

Date: _____

1	Never
2	Occasionally
3	Routinely
4	Not applicable

For each question below, circle the number to the right that best fits your operation. Answers should be for the **12 months**.
Group medication means >1 pig (this could include treating an entire pen or room).
Occasionally means treatment has been done in a disease outbreak situation and occurred in less than 30% of the pigs produced in the last year.
Routinely means treatment is done to prevent disease OR that more than 30% of animals produced in the last year have received treatment.

Group Medicating	Scale			
1. Suckling - Do you use group feed medication?	1	2	3	4
2. Suckling - Do you use group water medication?	1	2	3	4
3. Suckling - Do you use group injectable medication?	1	2	3	4
4. Nursery - Do you use group feed medication?	1	2	3	4
5. Nursery - Do you use group water medication?	1	2	3	4
6. Nursery - Do you use group injectable medication?	1	2	3	4
7. Grow-finish - Do you use group feed medication?	1	2	3	4
8. Grow-finish - Do you use group water medication?	1	2	3	4
9. Grow-finish - Do you use group injectable medication?	1	2	3	4
10. Replacement gilts - Do you use group feed medication?	1	2	3	4
11. Replacement gilts - Do you use group water medication?	1	2	3	4

12. Replacement gilts - Do you use group injectable medication?	1	2	3	4
13. Breeding herd - Do you use group feed medication?	1	2	3	4
14. Breeding herd - Do you use group water medication?	1	2	3	4
15. Breeding herd - Do you use group injectable medication?	1	2	3	4
16. Boars - Do you use group feed medication?	1	2	3	4
17. Boars - Do you use group water medication?	1	2	3	4
18. Boars - Do you use group injectable medication?	1	2	3	4

1	Never
2	Yes
3	Not applicable

Individual Animal Treatment	Scale		
19. Suckling - Do you use individual treatment?	1	2	3
20. Nursery - Do you use individual treatment?	1	2	3
20. Grower - Do you use individual treatment?	1	2	3
21. Finisher - Do you use individual treatment?	1	2	3
22. Replacement gilts - Do you use individual treatment?	1	2	3
23. Breeding Herd - Do you use individual treatment?	1	2	3
24. Boars - Do you use individual treatment?	1	2	3

25. Who makes treatment decisions on the farm day to day?
    a. Owner / manager
    b. Herdsman
    c. Veterinarian
    d. Other

26. Does your farm have a written treatment protocol? _____

    a. If yes, who wrote it? _____
    b. May we have a copy of it? _____

A.3 On-Farm In-Feed Antimicrobial Use

Herd ID _____

Stage of Production	Product Name and Manufacturer	Active Ingredient Name	Dose (ppm)	If not on label – intended dose above or below	Reason for Use Preventive or treatment: Enteric, Respiratory or Other	Duration (days administered) -may not be consecutive	In the last year, did ALL pigs for this stage receive the treatment? Yes or no	If no, number of animals that received treatment.

A.4 On-Farm Water Soluble Antimicrobial Use
Herd ID _____

Stage of Production	Product Name and Manufacturer	Active Ingredient Name	Intended dose and units	If not on label – intended dose above or below	Reason for Use; Preventive or Treatment and Enteric, Respiratory or Other	Duration (days administered) -may not be consecutive	In the last year, did ALL pigs for this stage receive the treatment? Yes or no	If no, number of animals that received treatment.

A.5 On-Farm Parenteral Antimicrobial Use
Herd ID_____

Production Phase: _____

To be used on farms without individual animal treatment records. Please fill out this form for every phase that does not have individual animal treatment records available.

1. Do you inject pigs with an antibiotic to *prevent* disease? Yes No
   i. If yes
      i. What % of the pigs are treated _____
      ii. What antibiotic is used _____
      iii. What dose (cc/kg or mg/kg) _____
      iv. Number of times per pig _____

2. Do you treat (inject) pigs that are sick? Yes No
   - If yes:
      i. Who makes daily treatment decisions on the farm? _____

3. Over the last year, what has been the most common disease in this production phase?
   i. Can you estimate how many pigs are typically treated for this problem? Any of the below "units" are fine.
      i. Treatments per day & average pigs at risk / day _____
      ii. Treatments per week & average pigs at risk / day _____
      iii. Treatments per batch or room (average pig/batch) _____
   ii. What antibiotic do you most commonly use to treat this? _____
   iii. What dose do you most commonly use? _____cc / Kg or mg/ kg
   iv. How many days do you treat for? _____
   v. If the drug is not effective, what is your second choice of antibiotic? _____
   vi. What dose do you most commonly use? _____cc/kg or mg/kg
   vii. How many days do you treat with the second drug for? _____

4. Over the last year, what has been the second most common disease in this production phase? _____
   i. Can you estimate how many pigs are typically treated for this problem? Any of the below "units" are fine.
      i. Treatments per day (get avg. # @ risk/ day) _____
      ii. Treatments per week(get avg. # @ risk/ day) _____
      iii. Treatments per batch or room (get avg. # @ risk) _____
   ii. What antibiotic do you most commonly use to treat this? _____
   iii. What dose do you most commonly use? _____cc / Kg or mg/ kg
   iv. How many days do you treat for? _____
   v. If the drug is not effective, what is your second choice of antibiotic? _____
   vi. What dose do you most commonly use? _____cc/kg or mg/kg

vii. How many days do you treat with the second drug for? _____

5. In an average week, day, or batch, how many piglets are treated with antibiotics? (& average number of pigs at risk)
   _____Units _____
6. What is the most commonly used antibiotic in this production phase?
   _____
7. What is the second most commonly used antibiotic in this production phase?
   _____

A.6 Animal Inventory
Herd ID _____

Breeding Stock

- Sow inventory May 2003 _____
- Sow inventory May 2004 _____
- Current number sows in barn _____
- Gilt inventory May 2003 _____
- Gilt inventory May 2004 _____
- Current number of gilts in barn _____

- Boar (including V-boar) inventory May 2003 _____
- Boar (including v-boar) inventory May 2004 _____
- Current number boars in barn _____

Suckling piglets

- Number of animals born alive May 2003 to May 2004 _____
- Number of animals weaned May 2003 to May 2004 _____
- Current number of suckling animals _____

Nursery

- Number of animals exiting nursery May 2003 to May 2004 _____
- Current number of nursery animals _____
- For farms that iso-wean – indicate number of days for each group in nursery and the % or number of animals in each group _____
  _____
  _____
  _____

Grow – finish inventory

- Number of animals produced May 2003 to May 2004 _____
- Current number of grow-finish animals _____

Breeding Companies

- Boars – number sold between May 2003 and May 2004 _____
- Average number of days in barn beyond when would typically have been sent for slaughter _____

- Gilts - number sold between May 2003 and May 2004 _____
- Average number of days in barn beyond when would typically have been sent for slaughter _____

A.7 Pig Characteristics by Production Phase

Production Phase	Days in phase	Age out	Weight out
Farrowing (weaning age)			
Nursery			
Grow-Finish			